UNIVERSIDADE DE LISBOA
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T Follicular Regulatory Cells in Human Adaptive Immunity and Autoimmunity

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Orientadores: Prof. Doutor Luís Ricardo Simões da Silva Graça
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Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, Imunologia.

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As opiniões expressas nesta publicação são da exclusiva responsabilidade do seu autor.

Para os quatro pilares da minha vida,

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In René Descartes, Discours de la méthode

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LIST OF ABBREVIATIONS

AID/Aicda	Activation-induced cytidine deaminase
AECG	American European consensus group
AIRE	Autoimmune regulator
AKT	Akt serine/threonine kinase
Alum	Aluminium
anti-dsDNA	Antibody anti-double strand deoxyribonucleic acid
anti-SSA/Ro52	Antibody anti-Sjögren syndrome-related antigen A/Ro52
anti-SSA/Ro60	Antibody anti-Sjögren syndrome-related antigen A/Ro60
anti-SSB/La	Antibody anti-Sjögren syndrome-related antigen B/La
AP Red	Red alkaline phosphatase substrate
APC	Antigen presenting cell
AS	Ankylosing spondylitis
Ascl2	Achaete-scute homologue-2
BATF	Basic leucine zipper transcription factor
BCA-1	B-cell-attracting chemokine 1
Bcl	Anti-apoptotic protein B-cell lymphoma
BCR	B-cell receptor
BLIMP-1/Prdm-1	PR domain zinc finger protein 1
BTK	Bruton's tyrosine kinase
BTLA	B and T-lymphocyte attenuator
BXD2/Tnfs11	Tumour necrosis factor (ligand) superfamily, member 11
C1q	Complement factor 1q
cAMP	Cyclic adenosine monophosphate
CARMA1	Caspase recruitment domain family member 11
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
Ciita	Class II major histocompatibility complex transactivator
c-Maf	bZIP transcription factor
Cre	Cre-recombinase enzyme

cREL	NF-kB subunit REL proto-oncogene
cTEC	Cortical thymic epithelial cells
CTLA-4/CD152	Cytotoxic T-lymphocyte-associated antigen 4
CTV	Cell trace violet
CVID	Common variable immunodeficiency
CXCL	CXC chemokine ligand
CXCR	CXC receptor
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DMARDs	Disease modifying anti-rheumatic drugs
DN	Double negative
DNA	Deoxyribonucleic acid
DNAse I	Deoxyribonuclease I
DOCK8	Dedicator of cytokinesis 8
DP	Double positive
DTR	Diphtheria toxin receptor
EBF	Early B-cell factor
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELS	Ectopic lymphoid structure
ERT	Oestrogen receptor induced by tamoxifen
ESSDAI	European league rheumatism Sjögren's syndrome disease activity score
ESSPRI	European league rheumatism Sjögren's syndrome patient reported index
FcγRIIB	Fc receptors IIB
FDC	Follicular dendritic cell
Flox	Floxed
Foxo	Forkhead box, subgroup O
Foxp	Forkhead box P
FSA	Focal sialadenitis
GATA	Glutamyl-tRNA amidotransferase, subunit A
GC	Germinal centre

GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumour necrosis receptor superfamily
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	Healthy donor
HEL	Hen egg lysozyme
Helios	IKAROS family zinc finger 2
HIER	Heat-induced epitopal retrieval
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
ICAM1	Intercellular adhesion molecule 1
ICOS	Inducible T-cell co-stimulator
ICOSL	ICOS ligand
Id	Inhibitor of DNA binging (HLH protein)
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IKKβ	Inhibitor of nuclear factor kappa B kinase subunit beta
IL	Interleukin
ILC	Innate lymphocyte cell
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRES	Internal ribosome entry sites
IRF	Interferon-regulatory factor
JAK	Janus kinase
KLF	Kruppel-like factor
KLH	Keyhole limper hemocyanin
LAG3	Lymphocyte activating 3
LCK	Src family tyrosine kinase proto-oncogene
LCMV	Lymphocytic choriomeningitis virus
LEF-1	Lymphoid enhancer binding factor 1
LPS	Lipopolysaccharide
LRBA	Lipopolysaccharide-responsive beige-like anchor protein

LTβ	Lymphotoxin beta
Ly108/SLAM6	SLAM family member 6
MALT	Mucosa-associated lymphoid tissue
MFGE8	Milk fat globule-EGF factor 8 protein
MFI	Mean fluorescence intensity
MG	Myasthenia gravis
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte
MS	Multiple Sclerosis
MSG	Minor salivary gland
mTEC	Medullar thymic epithelial cells
mTOR	Mechanistic target of rapamycin kinase
MYC	bHLH transcription factor proto-oncogene
Myd88	Myeloid differentiation primary response 88
NEMO	Inhibitor of nuclear factor kappa B kinase subunit gamma
NF-kB	Nuclear factor kappa B
non-SSS	Non-Sjögren's sicca syndrome
Notch	Neurogenic locus notch homolog
NP	4-hydroxy-3-nitrophenylacetyl hapten
OPN	Osteopontin
ORAI1	Calcium release-activated calcium modulator 1
OT-II	Anti-OVA TCR transgenic mice
OVA	Ovalbumin
OX40/CD134	Tumour necrosis factor receptor superfamily member 4
OX40L	OX40 ligand
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1/Pdcd1	Programmed cell death receptor 1
PD-L	PD-1 ligand
PI3K	Phosphatidylinositol-4,5-biphosphate 3-kinase
PKC	Protein kinase C
POLH	DNA polymerase eta
Pou2af1	POUC class 2 associating factor 1
PSGL-1/CD162	Selectin P ligand 1

pTreg	Peripherally-induced Treg cell
RA	Rheumatoid arthritis
Rag	Recombination-activating gene
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
RNASeq	Ribonucleic acid sequencing
RORγT	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute medium
Rptor	Regulatory associated protein of mTOR complex 1
RT-PCR	Real time PCR
RUNX3	Runt related transcription factor 3
SAP	SH2 domain containing 1A
SEA	*Staphylococcus aureus* enterotoxin A
SEB	*Staphylococcus aureus* enterotoxin B
SEE	*Staphylococcus aureus* enterotoxin E
SGEC	Salivary gland epithelial cells
SLAM	Signalling lymphocytic activation molecule
SLE	Systemic lupus erythematosus
SMARTA	TCR transgenic cells specific for LCMV
SOCE	Store-operated calcium entry
SP	Single positive
SRBC	Sheep red blood cells
SS	Sjögren's syndrome
STAT	Signal transducer and activator of transcription
STIM	Stromal interaction molecule
SYBR	SYBR green I, cyanine dye
T-bet	T-box 21
TC21	RAS related 2
TCF-1	HNF homeobox A
TCR	T-cell receptor
Tfh	T follicular helper cell
Tfr	T follicular regulatory cell
TGF-β	Transforming growth factor beta
Th	T helper cell

Thy	CD90
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TOX	Thymocyte selection associated high mobility group box
Tr1	T regulatory 1 cell
TRA	T-cell receptor alpha
TREC	T-cell receptor excision circles
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
TSST-1	Toxic shock syndrome toxin 1
tTreg	Thymic-derived Treg cell
WT	Wild type
Xbp1	X-box binding protein 1 pseudogene 1
YFP	Yellow fluorescent protein

SUMMARY

Germinal centres (GC) are formed during adaptive immune responses to defend our body against invading pathogens. During GC reactions antigen-specific high-affinity antibodies are produced through an intricated T – B cell crosstalk. In the last decades, the identification of a T cell subset specialized in controlling GC reactions, the T follicular helper (Tfh) cells, was a major scientific breakthrough. Within GCs, Tfh cells support B cell affinity maturation and class switch recombination. In addition, T follicular regulatory (Tfr) cells were recently described as GC regulators. Tfr cells are derived from thymic $Foxp3^+$ Treg cells and undergo a still poorly defined, Bcl-6-dependent, multistep differentiation pathway within secondary lymphoid tissues. Throughout this process, Tfr cells enforce tolerance and limit autoantibody-mediated autoimmune diseases by regulating Tfh – GC B cell interactions. Although the biology of human blood and tissue Tfh cells has been established, the biology and ontogeny of human blood Tfr cells, defined as $CXCR5^+Foxp3^+$ T cells, still remains elusive.

This work focused on Tfr cells in human adaptive immunity and autoimmunity. We have shown that human blood $CXCR5^+Foxp3^+$ Treg cells constitute a circulating counterpart of GC *bona fide* tissue Tfr cells. Indeed, those blood Tfr cells increase after the induction of GC responses by vaccination, and they are absent from human umbilical cord blood, where non-maternal foreign antigens are not present. However, blood Tfr cells are immature cells which fail to fully regulate humoral responses, suggesting these cells are not fully competent Tfr cells. To address the biological significance of blood Tfr cells in autoimmunity, we studied patients with a systemic autoimmune disease (Sjögren's syndrome) characterized by abnormal generation of autoantibodies within lymphoid structures ectopically formed in exocrine glands. Unexpectedly, Sjögren's syndrome patients had a striking increase in blood Tfr cells, as well as an increase in blood Tfr/Tfh ratio. In addition, we established the relationship between blood Tfr and Tfh cells and abnormal immune responses in the target organ of an autoimmune disease. Patients with ectopic lymphoid structures in their salivary glands had the highest blood Tfr/Tfh ratio, suggesting this ratio may be a novel biomarker of ectopic lymphoid activity in Sjögren syndrome.

Autoimmunity of follicular origin poses a considerable clinical challenge. Following the

discovery of Tfr cells as GC “fine tune” regulators, targeting Tfr cell responses may constitute a novel and highly selective approach for the treatment of autoantibody-mediated autoimmune diseases.

Keywords

Germinal centres; T follicular helper cells; T follicular regulatory cells; Autoimmunity; Sjögren syndrome.

SUMÁRIO

Durante as respostas imunológicas adaptativas formam-se centros germinativos, onde, através de interações celulares complexas, entre linfócitos T e linfócitos B, são produzidos anticorpos. Os anticorpos produzidos nos centros germinativos apresentam elevada afinidade e especificidade contra os antigénios que iniciaram a resposta imunológica, constituindo, por isso, um elemento essencial na defesa do organismo face a agentes invasores. Nas últimas décadas foi identificado o tipo de linfócitos T que, por interagir com linfócitos B de forma especializada, é responsável pela formação dos centros germinativos: as células T foliculares de ajuda (Tfh). No centro germinativo, as células Tfh são responsáveis pela maturação de afinidade e mudança de classe das imunoglobulinas dos linfócitos B. Recentemente foi descrito um outro tipo de linfócitos T especializado na regulação das interações entre as células Tfh e os linfócitos B que ocorrem nos centros germinativos, evitando a desregulação destas respostas e prevenindo o desenvolvimento de doenças autoimunes. Estes linfócitos designam-se células T foliculares reguladoras (Tfr). As células Tfr diferenciam-se nos órgãos linfoides secundários a partir de células T reguladoras formadas no timo e que expressam Foxp3. Esta via de diferenciação é ainda pouco conhecida, mas envolve vários processos que dependem da aquisição de expressão de Bcl-6 por parte das células T reguladoras. Contudo, o significado biológico e a função destas células (definidas como células T $CXCR5^{+}Foxp3^{+}$) no sangue dos seres humanos não é conhecido, contrariamente ao que acontece com as células Tfh, cuja biologia já foi estabelecida em humanos.

Este trabalho foi desenvolvido com o objetivo de estudar as células Tfr nas respostas imunológicas adaptativas (fisiológicas) e nas doenças autoimunes, em seres humanos. O trabalho experimental desenvolvido permitiu estabelecer, pela primeira vez, que as células T $CXCR5^{+}Foxp3^{+}$ existentes no sangue humano constituem um compartimento circulante das células Tfr formadas nos órgãos linfoides secundários. De facto, verificou-se um aumento destas células sanguíneas estas células após a vacinação e a sua ausência no sangue do cordão umbilical, onde não existem antigénios exógenos, para além dos maternos. Contudo, as células Tfr do sangue são imaturas e não são capazes de regular eficazmente a produção de anticorpos, pelo que as células T $CXCR5^{+}Foxp3^{+}$ do sangue humano não apresentam a mesma diferenciação funcional que as células Tfr dos órgãos linfoides

secundários. Para estudar a importância destas células nas doenças autoimunes estudaram-se doentes com síndrome de Sjögren. Esta doença autoimune sistémica é caracterizada pela formação ectópica de estruturas linfoides nas glândulas exócrinas. A produção de autoanticorpos ocorre maioritariamente nestas estruturas onde existem interações T – B patológicas. Contrariamente ao esperado, as células Tfr, descritas como apresentando funções reguladores, encontram-se aumentadas no sangue dos doentes com síndrome de Sjögren, tal como o rácio Tfr/Tfh. Este trabalho permitiu ainda estabelecer a relação entre as células Tfh e Tfr e os fenómenos imunológicos que ocorrem nos órgão-alvo desta doença autoimune. De facto, os doentes com formação ectópica de estruturas linfoides nas glândulas salivares são os que apresentam os valores mais elevados do rácio Tfr/Tfh, pelo que este rácio pode constituir um novo marcador para a identificação de doentes com formação ectópica de estruturas linfoides.

As doenças autoimunes cuja patogénese está relacionada com a perda de tolerância nos centros germinativos continuam a ser um desafio clinico e terapêutico. O desenvolvimento de fármacos capazes de modular a resposta células Tfr pode constituir uma nova abordagem terapêutica para estas doenças autoimunes.

Palavras-chave

Centros germinais; Células T foliculares de ajuda; Células T foliculares reguladoras; Autoimunidade; Síndrome de Sjögren.

SUMÁRIO EXTENSO

Apesar dos avanços científicos das últimas décadas, a complexidade dos mecanismos imunológicos que estão implicados na patogénese de várias doenças continua a ser um dos mais relevantes desafios da medicina moderna.

O sistema imunitário apresenta uma ambiguidade intrínseca: ao mesmo tempo que garante a proteção do organismo humano contra agentes invasores, através de mecanismos celulares e humorais potentes, o sistema imunitário integra mecanismos homeostáticos de tolerância para o *self*. A indução e manutenção de tolerância é, por isso, uma importante função do sistema imunitário, particularmente relevante na prevenção de respostas imunológicas inapropriadas contra o próprio organismo. As respostas humorais são um dos mais especializados e desenvolvidos mecanismos efetores do sistema imunitário. Estas respostas garantem a produção de anticorpos (específicos para o antigénio que iniciou a resposta) nos centros germinativos, através de interações celulares complexas entre linfócitos B e linfócitos T. As células T foliculares de ajuda (Tfh) constituem um tipo de linfócitos especializados na ativação e diferenciação de linfócitos B nos centros germinativos, sendo caracterizadas pela expressão do gene Bcl-6, do recetor CXCR5 (que permite a sua migração para a zona folicular dos órgãos linfoides) e moléculas diretamente envolvidas na estimulação de linfócitos B, tais como ICOS, CD40L e PD-1. A ausência das células Tfh é suficiente para impedir o estabelecimento de centros germinativos, constituindo uma causa de imunodeficiências primárias. Apesar da sua indiscutível importância na proteção contra agentes invasores, a desregulação das células Tfh pode culminar em respostas humorais excessivas capazes de promover o desenvolvimento de doenças autoimunes mediadas por autoanticorpos.

A manutenção da tolerância imunitária (e a consequente prevenção da autoimunidade) são uma das reconhecidas e amplamente estudadas funções das células T reguladoras (Treg), habitualmente definidas pela expressão do gene Foxp3. Recentemente foi identificado um subtipo de células Treg especializado na regulação das interações que ocorrem entre linfócitos B e células Tfh nos centros germinativos. Estas células T foliculares reguladoras (Tfr) expressam Foxp3 e diferenciam-se a partir de células Treg nos órgãos linfoides secundários, por mecanismos ainda pouco conhecidos. Durante a sua diferenciação as

células Tfr, tal como as células Tfh, adquirem a expressão de Bcl-6, CXCR5, PD-1 e ICOS, mas também a de CTLA-4 (um dos seus principais mecanismos efetores). Apesar do seu potencial papel na prevenção de doenças autoimunes mediadas por autoanticorpos, existem vários aspetos da biologia das células Tfr (definidas como células T $CXCR5^{+}Foxp3^{+}$) que não são conhecidos, sobretudo em seres humanos. O estudo desta população celular em seres humanos é de grande relevância antes de uma eventual translação terapêutica deste conhecimento. Contudo, o estudo das células Tfr em humanos tem sido limitado pelo difícil acesso a órgãos linfoides secundários (onde estas células se formam e exercem a sua função).

Este trabalho foi desenvolvido com o objetivo de estudar, em seres humanos, as células Tfr em respostas humorais (adaptativas) fisiológicas e em doenças autoimunes sistémicas.

Após a identificação das células Tfh nos órgãos linfoides secundários foi descrito um subtipo de linfócitos T circulantes com características fenotípicas e funcionais de células Tfh. Apesar de também terem sido identificados linfócitos T $CXCR5^{+}Foxp3^{+}$ no sangue humano (antes da primeira descrição das células Tfr) o seu significado biológico e a sua ontogenia não foram esclarecidos. Através da caracterização fenotípica de células T $CXCR5^{+}Foxp3^{+}$ em vários tecidos humanos e de ensaios funcionais demonstrou-se neste trabalho, pela primeira vez, que as células T $CXCR5^{+}Foxp3^{+}$ do sangue humano constituem um compartimento circulante das células Tfr formadas nos órgãos linfoides secundários. Esta conclusão fundamenta-se nas seguintes observações: a) a percentagem desta população celular no sangue aumenta após a vacinação contra o vírus da gripe; b) no sangue do cordão umbilical (onde não existem antigénios exógenos para além dos maternos) não existem células Tfr, embora se observem células Treg ativadas; c) estas células não existem no timo humano, pelo que a sua diferenciação ocorre nos órgãos linfoides periféricos; d) a estimulação *in vitro* com anti-CD3/CD28 não é suficiente para a expressão de CXCR5 pelas células Treg, demonstrando que a expressão de CXCR5 não é um mero marcador de ativação de células Treg (pelo contrário, a sua expressão é estável e funcionalmente relevante: as células Tfr são o único tipo de células Treg sanguíneas capazes de migrar a favor de um gradiente de CXCL13, responsável *in vivo* pela migração celular em direção às zonas foliculares); e, e) as células Tfr do sangue suprimem *in vitro* a proliferação de células Tfh. Contudo, contrariamente ao descrito para as células Tfr efetoras (presentes nos tecidos de ratinhos), o compartimento circulante não é capaz de suprimir completamente a produção de anticorpos (apesar de inibir a ativação de linfócitos B). De facto, as células Tfr do sangue humano são

imaturas (ou seja, não terminalmente diferenciadas), emergindo – dos tecidos linfoides – antes da interação com as células B, que é necessária para completar a sua diferenciação. Esta observação efetuada em doentes com uma imunodeficiência primária caracterizada pela ausência de células B demonstra que as células Tfr do sangue humanas não refletem a capacidade reguladora de respostas humorais do sistema imunitário.

Os mecanismos fisiopatológicos das doenças autoimunes sistémicas caracterizadas pela presença de autoanticorpos envolvem a perda de tolerância imunitária e podem, por isso, refletir respostas desreguladas de células Tfh e/ou Tfr. A síndrome de Sjögren é uma doença autoimune caracterizada por um processo inflamatório crónico das glândulas exócrinas (responsável pela xerostomia e xeroftalmia típicas desta doença) que pode atingir o sistema nervoso central, o rim e as vias respiratórias, acarretando ainda um risco significativo de evolução para linfoma. No contexto do processo inflamatório crónico desenvolvem-se, nas glândulas salivares, estruturas linfoides ectópicas, onde interações patológicas entre linfócitos T e linfócitos B culminam na produção de autoanticorpos. Para o diagnóstico da SS está recomendada a realização de uma biopsia das glândulas salivares, para além da pesquisa de autoanticorpos no sangue e de uma observação oftalmológica. Para estudar a relevância biológica e clínica das células Tfr do sangue nas doenças autoimunes foram colhidas amostras de sangue e de glândulas salivares de doentes com suspeita clínica de síndrome de Sjögren. Contrariamente ao esperado, as células Tfr, bem como o rácio Tfr/Tfh, encontram-se aumentadas no sangue dos doentes com síndrome de Sjögren (quando comparados com um grupo controlo recrutado simultaneamente). O aumento do rácio Tfr/Tfh correlaciona-se significativamente com a infiltração das glândulas salivares por linfócitos T e linfócitos B, permitindo discriminar os doentes com síndrome de Sjögren que apresentam a lesão histológica típica desta doença (designada sialadenite focal) dos doentes que não apresentam este achado histológico. Desta forma foi possível estabelecer, pela primeira vez, uma relação entre a população de células Tfr do sangue e a ocorrência de fenómenos imunológicos patológicos nos órgão-alvo de doenças autoimunes. Como se identificaram *in situ* células $CXCR5^{+}Foxp3^{+}$ nas lesões de sialadenite focal foi possível concluir que não existe exclusão anatómica destas células das estruturas linfoides ectópicas. estes locais. Por isso, integrando o conhecimento obtido quando se investigaram as células Tfr em respostas humorais fisiológicas, é possível que a perpetuação dos centros germinativos (um fenómeno reconhecido na autoimunidade) com respostas humorais persistentes e crónicas esteja na base do aumento destas células no sangue. Do ponto de vista

clínico, os resultados deste trabalho apontam para a utilização do rácio Tfr/Tfh do sangue humanos para identificar os doentes com síndrome de Sjögren que apresentam sialadenite focal – um fator prognóstico desta doença – sem recorrer à realização da biopsia.

As doenças autoimunes cuja patogénese está relacionada com a perda de tolerância nos centros germinativos continuam a ser um desafio clinico e terapêutico. O desenvolvimento de fármacos capazes de modular a resposta células Tfr pode constituir uma nova abordagem terapêutica para estas doenças autoimunes. O presente trabalho definiu o ontogenia e a biologia das células Tfr do sangue humano, permitindo, no futuro, que possam ser tentadas estratégias terapêuticas para modular estas respostas. Por outro lado, a utilização de um parâmetro não invasivo, que integra a informação sobre a frequência destas células no sangue humano – o rácio Tfr/Tfh – ao estratificar os doentes com SS que apresentam interações celular patológicas entre linfócitos T e linfócitos B nos órgãos-alvo da doença, pode ser útil na seleção dos melhores candidatos para as novas terapêuticas que se tem desenvolvido para modular estas interações celulares.

CHAPTER 1

GENERAL INTRODUCTION

1. The Immune System

Some of the greatest discoveries in life sciences and medicine arose from ethically questionable experiments. Edward Jenner launched *Modern Immunology* in such a way. He injected the material from a cowpox pustule into the arm of an 8-year-old boy. When this boy was later intentionally inoculated with smallpox, the disease did not develop. Janner's landmark treatise on vaccination was published in 1798 and led to the widespread acceptance of this method for inducing immunity to infectious diseases.

The immune system is historically regarded as the cells and molecules responding in a coordinated way to defend our body against invading threats. Cellular and biochemical defence mechanisms poised to respond rapidly and universally to invaders constitute the first line of defence, called *Innate Immunity*. Evolving during exposure to pathogens, the *Adaptive Immunity*, is characterized by the exquisitely ability to distinguish different substances (specificity), and to respond more vigorously to repeated exposures to the same pathogen (memory). Albeit immune responses were classically divided into two strands, the fundamental unity of immune responses is now widely recognized.

The cornerstones of the adaptive immune system are two broad sets of antigen-responsive cells: B and T lymphocytes. B lymphocytes are responsible for antibody production, using antibody in a membrane protein form as their antigen-binding receptors. Antibodies are capable of neutralizing pathogens (*Humoral Immunity*). By contrast, T cell receptors recognize a complex consisting of an antigen-derived peptide bound into a specialized groove in class I or class II major histocompatibility complex (MHC) molecules. As such, T cell recognition of antigen occurs on the surface of cells expressing these peptide/MHC complexes (*Cell-mediated Immunity*).

To fulfil the complexity of immune responses lymphoid tissues have evolutionally differentiated into highly organized structures, broadly classified as into primary and secondary lymphoid organs. *Primary lymphoid organs* comprise bone marrow and the thymus and are primarily responsible for the development of B and T lymphocytes. *Secondary lymphoid organs* (lymph nodes, spleen, and mucosal-associated lymphoid tissues) are critical for mounting high-affinity adaptive immune responses upon antigenic

challenge.

Lymphocytes are derived from pluripotent hematopoietic stem cells (HSC) present in the foetal liver and bone marrow. Common lymphoid progenitor derived from HSC give rise to lymphocytes committed to T cell lineage, under Notch-1 and GATA3 transcription factors, or to lymphocytes committed to B cell lineage, under EBF, E2A and Pax-5 transcription factors[1,2]. While B cell-committed lymphocyte precursors mature in the bone marrow (B-1 cells derived from foetal liver HSC and B-2 cells from bone marrow HSC), T cell-committed lymphocyte precursors circulate to the thymus, where they complete their maturation (γδ T cells from foetal liver HSC and αβ T cells from bone marrow HSC). Development of mature B and T cells is a complex process that contains numerous intrinsic steps at which the developing cells are tested and continue to mature only if a preceding step has been successfully completed. The rearrangement of antigen receptor genes (BCR in B cells and TCR in T cells), by V(D)J recombination[3], is the key event for maturation, as the correct rearrangement is essential for survival signals delivered to developing lymphocytes.

To mount high-affinity immune responses, B cells interact with T cells in the outer T cell zones of secondary lymphoid organs and differentiate along either the *follicular* or *extrafollicular* pathway. In the follicular pathway, activated B cells form germinal centres (GC) and exit these specialized structures as long-lived *antibody-producing plasma cells* or *memory B cells* that can respond and re-diversify to secondary challenges. In the extrafollicular pathway, B cells migrate to splenic bridging channels or junction zones and the borders between T cell zones and the red pulp, forming clusters of short-lived plasmablasts. The mechanisms responsible for this fate decision remain poorly defined, although various studies suggest that BCR affinity for the foreign antigen, the amount of antigen-receptor engagement, and costimulatory signals received from T cells might all be involved[4–8].

GCs are transient and specialized structures that form within secondary lymphoid tissues following follicular B cell differentiation. The GC was first described by Walther Flemming in 1884 as one of the major sources of lymphocytes throughout the body[9]. Although, his assumption proved to be wrong, GCs were found to be specialized structures where B cells expressing high-affinity antibodies develop and differentiate into antibody-secreting plasma cells and memory B cells under the critical help of T cells (T cell-dependent antibody

responses)[4,10,11]. Indeed, within GCs, B cells proliferate at a rate that is unparalleled in mammalian tissues and their immunoglobulin variable region genes are diversified by *somatic hypermutation*. This process results in the generation of mutant clones that have a broad range of affinity for the immunizing antigen. Throughout these processes of clonal proliferation, somatic hypermutation and selection, the affinity of B cell clones increases, in a phenomenon known as *affinity maturation*[6,12,13]. Although, GCs may not be an absolute requirement for affinity maturation, the selective advantage conferred by these specialized structures might be as an adaptation that supports a complex mechanism of cellular amplification and selection in response to antigens, while simultaneously limits the consequent risk of autoreactivity[14].

In 1968 a trio of publications by Miller and Mitchell described that GC B cell selection and antibody responses required matching thymus-derived T cell responses. Using cell transfer experiments, they showed that the co-transfer of both T and B cells to irradiated mice was absolutely necessary for robust antibody responses after immunization of mice with sheep red blood cells (SRBC)[10].

While the specialized formation of GC and T – B cell crosstalk are critical to provide protection against a broad range of invading pathogens, the stochastic nature of somatic hypermutation makes the generation of self-reactive B cell clones an almost certain by-product of routine GC responses to foreign antigens[15]. Failure of the immune system to enforce tolerance readily leads to the development of autoimmune disease and allergies. Therefore, the chief challenge of the immune system is to provide robust protection against pathogens while ensuring tolerance to self-antigens and innocuous non-self-antigens.

2. T Cell Mediated Humoral Immunity

2.1. Thymic T Cell Development

Development of T cells consists of several processes that require the dynamic relocation of developing lymphocytes into, within and out of multiple thymic environments, as well as an intricate crosstalk between T-cell committed lymphocytes, or thymocytes, and thymic

stromal cells[16,17]. Thymus seeding by lymphoid progenitor cells starts by the eight week of human gestation, initially by CCL21 and CCL25-mediated chemotactic attraction[18,19], and then through PSGL-1 and P-selectin[20]. Lymphoid progenitor cells begin their maturation as $CD4^-CD8^-$ double negative (DN) thymocytes. The cells that succeed in generating in-frame TCRβ rearrangement will assemble the pre-TCR complex, formed by TCRβ, pre-TCRα, CD3 and ζ proteins. The pre-TCR complex mediates the selection of developing DN thymocytes that productively rearranged β chain of the TCR. Along with the Delta-Notch interaction and signals delivered by IL-7[21,22], the successful expression of the pre-TCR, initiates recombination at the α chain of the TCR, and the signals for further transition to the $CD4^+CD8^+$ double positive (DP) stage.

DP thymocytes that are newly generated in the thymic cortex contain the unselected repertoire of T cells. DP thymocytes interact through their TCR with self-peptide-MHC complexes expressed by stromal cells (cTEC and dendritic cells). Following TCR recognition of peptide-MHC ligands at low avidity interactions, DP thymocytes are induced to receive signals for survival and further differentiation into single positive (SP) thymocytes. This process, called *positive selection*, enriches for "useful" T cells[17]. By contrast, high avidity interactions elicit signals that lead to deletion of thymocytes, by apoptosis, in a process called *negative selection*[17]. This process contributes to elimination of self-reactive T cells, thereby decreasing autoimmunity potential. Positively selected DP thymocytes are induced to differentiate into SP $CD4^+CD8^-$ (if TCR recognizes MHC class II molecules) or $CD4^-CD8^+$ (if TCR recognizes MHC class I molecules) thymocytes and relocate to the thymic medulla, throught CCR7-mediated chemotaxis towards medullary CCL19 and CCL21 gradients. The environmental cues, cellular signals and transcription factors involved in CD4/CD8-lineage choice are still being elucidated. The "kinetic signalling model" proposes that TCR-signalled DP thymocytes first terminate CD8 gene transcription and assess the effect of absent CD8 gene transcription on TCR signalling. If TCR-mediated positive selection signals persist in the absence of CD8 gene transcription (MHC class II signalling), thymocytes differentiate into $CD4^+$ T cells. If TCR-mediated positive selection signalling ceases in the absence of CD8 transcription (MHC class I signalling), thymocytes differentiate into $CD8^+$ T cells. Thus, the CD4/CD8-lineage choice is not a stochastic phenomenon, but a tightly regulated process by multiple clues, including IL-7 signalling and the orchestrated transcriptional activities of Th-POK, RUNX3, TOX and GATA3[23].

The final stage of thymocyte maturation is accompanied by further deletion of self-reactive thymocytes that have escaped negative selection in the cortex. Such additional deletion in the medulla seems to be particularly important in establishing *central tolerance* to tissue-specific antigens. This process is mediated by autoimmune regulator (AIRE)-dependent expression of tissue specific antigens by mTEC[24–26]. Central tolerance is also ensured by production of Foxp3-expressing regulatory T (Treg) cells in thymic medulla[27,28].

Mature naïve $CD4^+$ T cells and $CD8^+$ T cells egress from the thymus and circulate towards secondary lymphoid organs. After activation, effector $CD4^+$ T cells (Th cells) are mainly responsible for recruiting effector innate cells and for providing help to B cells leading to autoantibody production[29]. On the other hand, $CD8^+$ T cells (CTL cells) are mainly responsible for killing host-infected cells, through cytotoxic mechanisms[30].

2.2. $CD4^+$ T Cell Diversity

$CD4^+$ T cells constitute a highly heterogenous population of thymic derived lymphocytes. Besides cytokine-producing effector T-helper (Th) cells derived from naïve $CD4^+$ T cells, $CD4^+$ T cells also comprise thymic derived (or naturally occurring) Foxp3-expressing Treg cells.

The process of forming effector $CD4^+$ T cells begins when the TCR on naïve $CD4^+$ T cells recognises peptide-MHCII complexes on dendritic cells (DCs) in secondary lymphoid organs. Signals through TCR and antigen-presenting cell-derived costimulatory molecules (namely, CD80/CD86) trigger the naïve cells to divide and became effector cells, as postulated by Burnet's clonal selection theory[31,32]. Depending on the nature of cytokines produced by the innate immune system, inexperienced $CD4^+$ T cells undergo a differentiation process toward distinct Th lineages able to tailor their responses to the character of the threat encountered[29]. The initial insights for such diversity came from studies examining antigen-specific T cell clones from immunized mice[33,34]. It was postulated that Th1 cells orchestrated a phagocytic and intracellular defence, and Th2 cells orchestrated a nonphagocytic and extracellular defence. This binary division of labour was proved to be wrong after the discovery of Th17 cells[35,36].

Briefly, INF-γ and IL-12 secretion by DCs promote Th1 program in naïve T cells by inducing the expression of T-bet through STAT4 signaling[29,37–40]. On the other hand, IL-4 secretion by DCs induces GATA3 expression through STAT6 signalling on naïve T cells[29,40–44]. These Th2 cells will became specialized producers of IL-4, IL-5 and IL-13. IL-17-producing Th17 fate is instructed by upregulation of RORγT, by TGF-β, IL-6, IL-23 and IL-21 signalling (through STAT3)[29,40,45–48]. Although this paradigm was a useful construct for understanding cell-mediated immunity, flexibility and plasticity of $CD4^+$ T cells is now widely accepted[40].

More recently, a $CD4^+$ T cell subset specialized in B cell-helping functions was identified. These cells upregulate Bcl-6 (through STAT3 signalling) becoming IL-21-producing Tfh cells.

2.3. T Follicular Helper Cells

A $CD4^+$ T cell subset specialized in B cell help was first proposed at the turn of the millenium[49–51]. Schaerli, Breitfeld and Kim described a subset of CXCR5-expressing T cells in human tonsils co-localized with mantle and light-zone B cells. Using *in vitro* assays, they showed that $CXCR5^+CD4^+$ T cells selectively migrated toward a CXCL13/BCA-1 gradient, secreted IL-4 and IL-10 (but not other Th1/Th2 cytokines) and specifically promoted IgA and IgG production by B cells. These T helper cells were highly prone to apoptosis as almost all cells expressed Fas/CD95[49]. The impact of $CXCR5^+CD4^+$ T cells in IgM production was more controversial in these pioneering studies, as only two studies demonstrated a significant increase in IgM secretion by B cells co-cultured with $CXCR5^+CD4^+$ T cells compared to $CXCR5^-CD4^+$ T cells[49,50]. Additionally, a subset of CXCR5-exppressing $CD4^+$ T cells was identified in peripheral blood with the capacity to proliferate in response to allogenic macrophages, but these blood cells did not prove to be specialized in B cell help as compared to blood $CXCR5^-CD4^+$ T cells. Conversely, in light of more recent and robust evidence, blood $CXCR5^+CD4^+$ T cells were also found to be capable of providing help to B cells (see below)[52–56].

Subsequent studies showed that GC T cells in human tonsils highly expressed Bcl-6, providing the first clue of a lineage defining transcription factor[57]. In fact, the existence of Tfh cells was only widely accepted after BCL-6 was identified as a lineage-defining transcription factor of Tfh cells[58–60]. A range of experiments – including the use of $Bcl6^{-/-}$

$CD4^+$ T cells, constitutive expression of BCL-6 in antigen-specific $CD4^+$ T cells, and manipulation of the expression of Blimp-1 (a potent antagonist of Bcl-6) – showed that the expression of Bcl-6 by $CD4^+$ T cells is necessary and sufficient for Tfh cell differentiation and that Tfh cells are the unique providers of T cell help to B cell for the generation of most high affinity class-switched antibodies. Moreover, Bcl-6 expression was found to be induced by IL-6 and IL-21 and to drive Tfh cell differentiation, as it promotes upregulation of CXCR5, ICOS and PD-1[59–61]. Notably, Bcl-6 is not only required for the multifaceted Tfh cell biology, but also to repress alternative Th1, Th2 and Th17 cell fates[60,62].

Following the identification of Bcl-6, the study of Tfh cells has markedly increased. Stages of Tfh cell differentiation, inductive signals, migration patterns, Tfh cell memory and plasticity, and Tfh cell function have been extensively studied[63].

Antigen presentation by MHCII-expressing cells is absolutely required for priming naïve $CD4^+$ T cells and initiating Th subset differentiation. Likewise, Tfh cells require antigen presentation by DCs for their differentiation[64]. DCs induce Tfh cell differentiation through antigen presentation, co-stimulation through CD80/86:CD28, ICOSL:ICOS, and OX40L:OX40 signalling, and by generating a favourable cytokine milieu. Compared to Th1, Th2, and Th17 cells, the differentiation of Tfh cells requires persistent TCR stimulation[64,65]. Moreover, TCR dwell time on peptide:MHCII complex may also instruct the fate of naïve T cells, as naïve T cells with longer TCR-peptide:MHCII dwell time preferentially differentiate into Tfh cells[66,67]. CD28 and ICOS signalling are both critical checkpoints during Tfh cell differentiation[68–72]. The unique dependence of Tfh cells on ICOS co-stimulation is thought to be mediated through PI3K signalling. Activation of PI3K-Akt pathway by ICOS engagement phosphorylates Foxo1 and leads to its nuclear exportation and degradation. Foxo1 inactivation removes its repression of Bcl-6 expression, and its activation of KLF2 expression, thus promoting Tfh cell formation[70,73,74]. Additionally, these co-stimulatory molecules are also important for Tfh cell maintenance[69,74]. Besides CD28 and ICOS, OX40 was also shown to amplify Tfh cell development and GC responses, by cooperating with ICOS[75,76]. A suitable cytokine microenvironment is also essential for Tfh cell differentiation. IL-6 secreted by DCs potentiates Tfh cell lineage commitment and differentiation through STAT1 and STAT3 pathways, although other factors, such as high IL-21 and low IL-2, are also required[77–80]. While, IL-21 have a redundant action with IL-6, the former was shown to contribute to an autocrine regulation of Tfh cell formation[61,81,82].

Among all cytokines which orchestrate GC responses, IL-2 has been regarded as one key regulator of T cell progenies upon DC priming. Indeed, low IL-2 concentration is required to maintain Bcl-6-dependent transcriptional program in developing Tfh cell and to allow GC formation, as IL-2 suppress Bcl-6 and induce Blimp-1 (via STAT5 activation)[83–86]. Thus, Tfh cell development may not rely so much on a particular set of instructions from DCs. Instead, their development may depend more on a chance escape from a IL-2 enriched environment that favours terminal Th effector cell differentiation combined with a chance encounter with B-cell follicle environment[83]. Interestingly, some activated DCs were found to be able to quench T-cell-derived IL-2 by producing membrane bound and soluble CD25 (the high affinity Il-2 receptor), thus favouring Tfh cell differentiation[87].

Although, DC role in Tfh cell differentiation is unquestionable, DC priming alone is not sufficient to induce fully competent Tfh cells. Indeed, developing Tfh cells require sequential antigen presentation and cell-to-cell interactions with cognate B cells at the T-B border[64,88]. Following the initial DC priming, developing Tfh cells migrate to the T-B border and form cognate T-B cell conjugates. These conjugates are stabilized by homotypic interactions between the SLAM family members CD84 and Ly108, which thereafter signal via SAP[89,90]. In the absence of SAP, or antigen-presenting cognate B cells, GC Tfh cells do not form, demonstrating the absolute requirement for cognate T-B interactions in full development of Tfh cells[63,89,90]. At this stage of the immune response, activated B cells act as the dominant antigen presenting cell (APC) further supporting Tfh cell development through CD80/CD86, ICOSL, and CD40 co-stimulatory signals[61,64,69,91,92]. Recently, Watanabe and colleagues found that while CD80/CD86 was required on DCs, CD40 was required on B cells for the generation of antigen-specific Tfh cells[92]. Additionally, bystander B cells may act independently of cognate interactions, by providing ICOSL to developing Tfh cells, thereby promoting their migration toward the B cell follicle[72].

All these biological processes shape the transcriptional profile of a naïve T cell toward a distinctive Tfh cell gene profile. In several murine studies it was established that Bcl-6, c-Maf, BATF, IRF4, STAT1, STAT3, STAT4, Notch1, Notch2, Ascl2, TCF-1, and LEF-1 positively influence Tfh cell program; while Foxo1, Foxp1, Blimp1, KLF2, negatively influence Tfh cell program[58–61,63,73,74,79,86,93–103]. This molecular machinery is responsible for the acquisition of specific migration capacity toward B cell follicles on secondary lymphoid tissues and of specialized effector functions by developing Tfh cells. Ascl2 and Bcl-6 (via

Blimp-1 repression) induce CXCR5 upregulation which, along with downregulation of CCR7 directly by Bcl-6, promotes Tfh cell migration toward CXCL13-abundant follicles[62,98,104,105]. As described above, Tfh cells get access to GC light zone after previous interactions with cognate B cells at the T-B border. Within GCs the provision of help from Tfh cells to GC B cells comes in the form of CD40 ligation and cytokine signals, namely IL-21[63]. Interaction with CD40L is required for maintenance of established GC responses, continued survival of GC B cells, and formation of high-affinity bone marrow plasma cells[106–110]. Tfh cell-derived IL-21, by maintaining Bcl-6 expression, in GC B cells, controls the maintenance and optimal affinity maturation of the GC response[111,112]. Besides IL-21, Tfh cells can shape their cytokine secretion accordingly with the stimulus that induced the GC reaction. Tfh cells were found to secrete IL-4 in type 2 induced responses and IFN-γ in type 1 induced responses, thereby modulating class switch of B cells toward IgG1 and IgG2a, respectively[113–115]. It is still debatable whether Tfh cells modulates class switch recombination taking advantage of cytokines produced by Th cells outside the GC or two distinct IFN-γ-producing and IL-4-producing Tfh cells directly modulate class switch recombination in type 1 and type 2 immune responses, respectively. Our group is currently approaching this important question using RNA-Seq data generated from sorted TCR-specific Tfh cells differentiated under type 1 and type 2 conditions.

2.3.1. Circulating Tfh Cells and Human-Specific Tfh Cell Features

Tfh cell discovery remarkably uncovered the biology of $CXCR5^+CD4^+$ T cells in human blood, known since 1994[116]. Several independent observations have recently established that Tfh-committed cells can egress from lymph nodes comprising a memory compartment of Tfh lineage cells[52–56]. The concept that blood $CXCR5^+CD4^+$ T cells are generated from cells committed to Tfh fate is based on two main lines of evidence.

First, blood $CXCR5^+CD4^+$ T cells were shown to selectively promote *in vitro* activation and class switch recombination of B cells[53–56]. Chevalier and colleagues found that $CXCR5^+$ T cells sorted from human blood were more efficient at providing B cell help (compared to $CXCR5^-$ T cells), as a higher proportion of activated B cells, as well as higher supernatant IgA and IgG concentrations, were found in co-cultures upon superantigen stimulation[53]. $CXCR5^+$ T cells provided help to B cells in a ICOS and IL-10-dependent manner, as the use of ICOS and IL-10 blocking antibodies abrogated the secretion of IgA and IgM by activated

B cells[53]. In those experimental conditions, CXCR5$^+$ T cells secreted high amounts of IL-17, IL-10, and IL-21[53,82,111,112]. Moreover, CXCR5$^+$ T cells were found to upregulate Bcl-6 and c-Maf upon in a greater extended than in CXCR5$^-$ T cells upon *in vitro* stimulation[53]. In another report, Morita and colleagues clearly showed human blood CXCR5$^+$ T cells were necessary and sufficient to induce activation-induced cytidine deaminase (AID) upregulation and Ig secretion by B cells, which survive and upregulate activation markers (such as CD38) upon *in vitro* stimulation with superantigens and CXCR5$^+$ T cells, but not with CXCR5$^-$ T cells[54]. They further validated that human blood CXCR5$^+$ T cells produce IL-21 and IL-10 upon superantigen stimulation[54]. Furthermore, IgA and IgM titres in co-culture supernatants markedly decreased when IL-21-blocking antibodies were used[54]. The use of superantigens (such as SEA, SEB, SEE and TSST-1) greatly improved the experimental design to study T-B interactions, as superantigens promote cognate interactions more similar to physiological cell interaction *in vivo*[52]. The second evidence relies on studies with blood samples from primary immunodeficient patients with severely impaired GC formation due to deficiency of IL-10R, CD40L, NEMO, BTK, ICOS, IL-12R, STAT3 loss of function; presenting also a profound and selective reduction of blood CXCR5$^+$CD4$^+$ T cells[71,117–119].

Although, this compelling evidence suggests human blood CXCR5$^+$ T cells are counterparts of *bona fide* GC Tfh cells, there are many differences between these two cell populations. Whereas, the vast majority of blood Tfh cells express CD45RO, CD62L and CCR7, tissue Tfh cells downregulate CCR7 to gain access to GC, as described above[49–51,105]. In great contrast to GC Tfh cells, most blood Tfh cells are in a quiescent state and lack the expression of Bcl-6, PD-1 and ICOS, suggesting that Bcl-6-dependent transcriptional program is dispensable for the maintenance of blood memory Tfh cells[52,54,56,120]. Hence, human blood Tfh cells express elevated levels of c-Maf, suggesting that while Bcl-6 is required for Tfh cell differentiation, c-Maf is required for the maintenance of Tfh cell phenotype and/or function[53,55,121].

Human Blood Tfh Cell Diversity

Extensive analysis of human blood Tfh cells have further revealed phenotypic and functionally distinct subsets (**Table 1**).

As described above, more than 70% of human blood CXCR5$^+$CD45RO$^+$ Tfh cells are Ki-

67$^-$ quiescent cells which lack expression of ICOS and PD-1, while expressing CCR7[52,55,56]. He and colleagues reported that blood Tfh cells are heterogeneous and are composed of CCR7$^+$PD-1$^-$ and CCR7$^-$PD-1$^+$ subsets. These two subsets showed distinct kinetics following seasonal influenza vaccination, as only CCR7$^-$PD-1$^+$ Tfh cells increased upon vaccination[56]. By comparing these results with mice transferred with congenic marked Ovalbumin (OVA)-specific OT-II cells and then immunized with OVA in alum, He and colleagues proposed that CCR7$^-$PD-1$^+$ Tfh cells reflect tissue Tfh cell responses[56]. Additionally, they demonstrated that CXCR5$^+$CCR7$^-$ Tfh cells sorted from human blood were more efficient in supporting plasma cell differentiation and IgG production, compared to CXCR5$^+$CCR7$^+$ Tfh cells, upon *in vitro* TCR stimulation[56]. Using a different experimental design, Morita and colleagues demonstrated that human blood CXCR5$^+$ Tfh cells were composed of three subsets according to expression of CXCR3 and CCR6 chemokine receptors[54]. The T-bet$^+$CXCR3$^+$CCR6$^-$ Tfh1, GATA-3$^+$CXCR3$^-$CCR6$^-$ Tfh2, and RORγt$^+$CXCR3$^-$CCR6$^+$ Tfh17-like cells were proved functionally distinct[54]. Whereas Tfh1, Tfh2 and Tfh17-like cells were able to secrete the defining cytokines of their Th counterparts, namely IFN-γ (Tfh1), IL-4, IL-5 and IL-13 (Tfh2), and IL-17 and IL-22 (Tfh17), only Tfh2 and Tfh17-like cells produced IL-21, induced activation of naïve B cells, and supported class switch recombination upon *in vitro* stimulation with superantigens[54]. This was further confirmed in GC Tfh cells from human tonsils in which T-bet was found to diminish the Tfh cell program required to provide help to B cells[122]. Taking advantage of these findings, Locci and colleagues used gene expression microarrays to demonstrate that PD-1$^+$CXCR3$^-$ Tfh cells were the blood Tfh cell subset which most closely resemble GC Tfh cells (sorted from human tonsils)[55]. Using *in vitro* co-cultures under the presence of superantigens, PD-1$^+$CXCR3$^-$ Tfh cells displayed the strongest capacity to induce IgG and IgM secretion by memory B cells[55].

The clinical relevance of blood Tfh cell subsets is supported by vaccination studies. Following seasonal influenza vaccination, the emergence of blood ICOS$^+$CXCR3$^+$ Tfh cells correlated with the development of protective antibodies[120,123]. Thus, Tfh cell subsets could serve as potential biomarkers for monitoring antibody responses in vaccination and infectious diseases. However, it is somehow unexpected that Bentebibel and colleagues correlated blood CXCR3$^+$ Tfh cells with humoral responses. Indeed, CXCR3-expressing Tfh cells were previously shown to be non-efficient helpers[54,55,122]. The help activity of blood ICOS$^+$CXCR3$^+$ Tfh cells was limited to memory B cells (and not to naïve B cells) upon *in*

vitro stimulation with superantigens[120]. Therefore, it is likely that ICOS$^+$CXCR3$^+$ Tfh cells specifically evoke memory humoral responses which bypass *de novo* GC reactions.

Table 1: Human blood Tfh cell subsets
Adapted from Schmitt *at al*[52]

		Follicular (activation) markers	
		ICOS$^-$ PD-1$^-$	ICOS$^+$ PD-1$^+$
Chemokine Receptors	CXCR3$^+$ CCR6$^-$	**Quiescent Tfh1**	**Activated Tfh1**
	CXCR3$^-$ CCR6$^-$	**Quiescent Tfh2**	**Activated Tfh2**
	CXCR3$^-$ CCR6$^+$	**Quiescent Tfh17**	**Activated Tfh17**

Distinctive Features of Human Tfh Cells

Although, several studies have established that Tfh cells from humans and mice are similar at the transcription level, a parallel body of work implies a largely different group of cytokines in human Tfh cell differentiation[62,117,121,124–128]. Schmitt and colleagues systematically analysed the upregulation of Tfh-signature molecules by sorted naïve CD4$^+$ T cells from human blood cultured in the presence of several combinations of cytokines plus anti-CD3/CD28 stimulation[126]. TGF-β plus IL-12 or TGF-β plus IL-23 induced upregulation of CXCR5, ICOS, Bcl-6, and downregulation of Blimp-1. These cytokine combinations also increased the proportion of IL-21-expressing T cells in the end of culture and efficiently enabled those T cells to provide help to memory B cells, measured by IgG concentration in culture supernatants[126]. These findings suggest that TGF-β co-opts redundant signalling via STAT3/STAT4 (induced by IL-12 and IL-23) to promote Tfh cell differentiation in humans. While, preformed in artificial systems, the reduced Tfh cell and GC responses observed in patients lacking functional IL-12Rβ1, the receptor for IL-12 and IL-23, corroborates the IL-12 requirement for human Tfh cell differentiation in physiological conditions[117].

3. Regulation of Humoral Responses

To ensure clonal selection of high affinity B cell clones, GC reaction is tightly regulated by complex molecular signals at multiple stages.

3.1. Germinal Centre Reaction and Cell Dynamics

Following immunization, IgD^+ naïve B cells are activated and move to the outer follicular zone of secondary lymphoid organs. Here, they present antigenic peptides on MHCII to specialized subsets of separately-activated $CD4^+$ T helper cells[5,129]. The antigen-activated B cells harbouring the highest BCR affinity for the immunizing antigen eventually gain access to the follicle centre and populate the specialized follicular dendritic cell (FDC) network[5,6,130]. After these initial steps of GC reaction, two main compartments become evident: the dark and light zones, as initially proposed by Röhlich in 1930. The *dark zone* is formed by densely packed B cells (classically called centroblasts) undergoing rapid proliferation and somatic hypermutation. This Darwinian process results in the generation of B cell clones (classically termed centrocytes) expressing surface antibodies with a wide range of affinities for the immunizing antigen. These cells then travel to the *light zone* where they compete for binding to immune complexes presented by highly ordered surface units of FDC (termed iccosomes), and for survival and differentiation signals provided by T helper cells[4,63,131–135]. During the course of cognate T-B cell interaction in the light zone, a subset of B cells additionally undergo activation-induced cytidine deaminase (AID)-mediated *class switch recombination*[136,137]. A great work by Victora and colleagues confirmed that T cell help, and not direct competition for antigen is the limiting factor for B cell selection within GCs[135,138]. Therefore, it seems that within the context of the GC , the role of the BCR is primarily to capture and internalize antigen rather than to induce BCR signalling[6,135]. Ultimately, high-affinity GC B cell clones differentiate into plasmablasts and memory B cells after repeated rounds of expansion, diversification and selection. This cyclic re-entry sustains the population of cells that proliferate within the dark zone and maintains the GC reaction over time[5,6,136]. Although, the precise mechanisms driving the fate decision of GC B cells are still being elucidated, Tfh cell help was found to favour plasma cell differentiation (and not memory B cell differentiation) in a CD40L-dependent manner[108–110,139,140].

The dark and light zones of the GC are organized by expression of CXCR4 and CXCR5, respectively. These chemokine receptors on B cells are required to guide B cell migration toward CXCL12-enriched dark zone (populated by $CXCR4^{hi}$ B cells) and CXCL13-enriched light zone (populated by $CXCR4^{lo}$ B cells)[131,135]. These phenotypic states are the result of a timed B cell-intrinsic program: Bcl-6 is required for the establishment of the specialized dark zone-associated gene expression programme that allows somatic hypermutation, MYC and

REL are required for recirculation between dark and light zones, and IRF4 is required for class switch recombination and plasma cell differentiation in the light zone[5,6,136,141]. Recently, McHeyzer-Williams and colleagues proposed that CD83 (required for cognate T-B interaction in the light zone) and POLH (which encodes the DNA polymerase required for somatic hypermutation in the dark zone) expression defines four sequential and cyclic GC B cell-stages[5,142]. Overall, like Ian MacLennan proposed in 1994, GC is functionally polarized into a dark zone in which B cells divide and a light zone in which B cells are activated and selected based on their affinity for antigen (via Tfh cells). Rather than differentiating linearly from centroblast to centrocytes and then to plasma or memory B cells, GC B cells are constantly shifting between two closely related but functionally distinct states, which are associated with positioning in either dark or light zones[132,139].

Throughout this process, BCR signalling, FDC and Tfh cells derived signals, as well as antigen immunogenicity, availability and clearance all contribute to maintain GC tolerance (**Table 2**). However, an additional regulatory cell mechanism is required to ensure systemic tolerance and to control humoral responses.

Table 2: Mechanisms that control germinal centre B cells responses
Adapted from Vinuesa *at al*[143]

Germinal centre component	Control of quality	Control of quantity
B cells	– Positive selection: BCR signals (TC21, PI3K, CD45 and SPIB), B cell – FDC synapse (DOCK8 and ICAM1) and Tfh cell derived signals (CD40L and IL-21) – Negative selection: Tfh cell signals (CD95L)	– Development and proliferation: IRF8 and PU.1, Bcl-6, IL-21R, TLRs, and Myd88 – Pro-apoptotic programme: high AID, CD95 and BIM, and low Bcl-2 and Bcl-X_L
Antigen	– Immunogenicity and availability: post-translational modification, structural features, development isoforms and abundant TLR ligands exposed on apoptotic cells.	– Genetic differences in expression: HLA and antigen gene variants – Disposol of apoptotic cells by tangible body macrophages: MFGE8, DNaseI, MER and C1q

3.2. Regulatory T Cells

Negative selection in the thymus leads to elimination or inactivation of self-reactive T cells[17]. In the periphery, the requirement of simultaneous engagement of TCRs by a cognate peptide-MHC complex and the T cell costimulatory receptor CD28 by CD80/CD86 reinforces self-tolerance[144]. However, these tolerance mechanisms appear insufficient to

counter the threat of immune-mediated pathology without a specialized subset of T cells acting to restrain immune responses towards self.

The existence of thymic generated T cells involved in immune tolerance were first revealed in neonatal thymectomy mice studies performed by Nishizuba, back in 1969[145]. This specialized subset of T cells (Treg cells) engaged with suppressive functions was later found to constitutively express IL-2 receptor α-chain (CD25)[146]. However, a seminal advance in the understanting of tolerance induced by Treg cells came with the identification of and the X-linked transcription factor forkhead box P3 (Foxp3) by Hori and colleagues in 2002[147–149]. Fontenot and colleagues generated mixed bone marrow chimeras by reconstituting lethally irradiated mice with T cell-depleted bone marrow mixed at 1:1 ratio with bone marrow from either Foxp3-sufficient and -deficient mice. Treg cells were generated only from Foxp3-sufficient precursors, clearly demonstrating the requirement of Foxp3 for the development of Treg cells[148]. Foxp3 is not only an absolute requirement for thymic differentiation of Tregs, but also for their suppressive function, as retroviral transfer of Foxp3 gene into $CD25^{-}CD4^{+}$ T cells confers suppressive properties and Cre-mediated ablation of conditional Foxp3 allele in Treg cells results in a loss of suppressive function[148,150]. Subsequent studies have also identified similar Foxp3-expressing Treg cells in human blood and lymphoid tissues[151].

The importance of Treg cells on the control of antibody responses has been long known[152–155]. Indeed, mice and humans with loss-of-function mutation in the Foxp3 gene suffer from a fatal, early-onset, T cell-dependent, lymphoproliferative, immune-mediated disorder manifested by autoantibody-mediated autoimmunity (diabetes, thyroiditis, haemolytic anaemia) and increased levels of circulating GC-produced antibodies, namely IgG and IgE, called immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans and scurfy condition in mice[156–160]. Moreover, chronic ablation of Treg cells in adult healthy $Foxp3^{DTR}$ mice caused a scurfy-like disease, which can be rescued by adoptive cell transfer of Treg cells[148,161].

Further studies elucidated the mechanisms of humoral suppression by Treg cells. Lim and colleagues have established the existence of a $CD69^{-}$ human tonsil Treg cell subset with B cell suppressive function[162,163]. Upon activation, this subset upregulated CXCR5, acquiring the capacity to migrate towards CXCL13-enriched GC. In their *in vitro* assays, Lim and

colleagues demonstrated that these cells suppressed class-switch recombination by B cells, as they inhibited immunoglobulin production and AID expression. Direct killing of B-cells by mice pre-activated (by anti-CD3 TCR stimulation) $CD4^+CD25^+$ T cells was also demonstrated[164]. This effect was mediated by a granzyme B-dependent, partially perforin-dependent, apoptotic pathway. Interestingly, antigen-pulsed B cells were more susceptible to death, suggesting a non-random regulatory mechanism[164]. These findings were further confirmed in a Systemic Lupus Erythematosus (SLE) mousee model and in SLE patients. Treg cells sorted from SLE patients inhibited the secretion of IgG by inducing B cell apoptosis, under the presence of CD40L, IL-4, and IL-10[165].

In addition to thymic derived Treg (tTreg) cells, many studies have identified Foxp3-expressing Treg cells induced from naïve $CD4^+$ T cells in peripheral tissues, called peripherally-derived Treg (pTreg) cells. While the mechanisms and complexity of pTreg cells are still not fully understood pTreg cells are generated following specific (non-self) antigen stimulation in particular environments and under the presence of TGF-β and IL-2[28]. Additionally to Foxp3-expressing Treg cells, IL-10 producing Treg (Tr1) and TGFβ producing Treg (Th3) cells, which work in a cell contact independent manner to induce tolerance to foreign antigens in specific tissue environments, have also been described[166–170]. Albeit identified in humans, the biological relevance of Tr1 and Th3 cells for human immune tolerance remains largely undefined[29,167,168].

Thymic Development of Treg Cells

Thymic development of Treg cells requires two steps. First, high-affinity interactions between TCR of $CD4^+CD8^-$ SP thymocytes and self-peptide-MHCII complexes presented by thymic stromal cells, as well as costimulatory signals (namely CD28 and CD40) takes place (*TCR-dependent step*). After this stage, $Foxp3^-CD25^+CD4^+CD8^-$ tTreg cell precursors will require further stimulation by IL-2, IL-7 and IL-15 to fully express Foxp3 and differentiate into tTregs (*TCR-independent step*)[27,28,171,172].

During thymic development the emergence of $CD4^+CD8^+$ DP thymocytes with self-reactive TCR is required for the differentiation of tTreg cells[172,173]. Compelling evidence suggests that thymic Treg cell development occurs when TCR avidity for self-antigens lies between the TCR avidities that drive positive selection and negative selection[28,172]. In addition to the requirement for distinct TCR specificities[174–176], thymic Treg cell development is typically

governed by intraclonal competition for an antigen-specific niche, suggesting that antigens themselves are likely to be rare and tissue specific[172,177]. Assuming TCR self-reactivity drives the thymic selection of Treg cells based on TCR signal strength (which broadly encompasses affinity, avidity and antigen-APC availability), molecular signals downstream of the TCR will further guide the development of Treg cells. After TCR engagement a variety of downstream molecules, including AKT, mTOR, NFAT and NF-kB will link TCR signals to Foxp3 transcription[172]. NF-kB pathway appears to be the main downstream pathway guiding thymic development of Treg cells, as mice with mutations in genes encoding components of this signalling cascade, such as PKCθ, CARMA1, Bcl-10, TAKI, IKKβ, and cREL, displayed dramatic decreases in the frequency of tTreg cells[28,172,178–181]. While, NF-kB pathways leads to Foxp3 expression, AKT-mTOR is inhibitory to differentiation of Tregs, via inhibition of FOXO1 and FOXO3, which have recently been shown to be required for thymic development of Treg cells[28,172,182–184]. Although, CD28-deficient mice have a dramatic reduction in the frequency of tTreg cells, recent evidence suggests that the primary role of CD28 signals (as well as of CD40 signals), is to enhance either the efficiency of development and/or the survival of Tregs, and not to promote selection of thymocytes into the lineage of Foxp3-expressing cells[28,172,185–187]. During TCR-dependent selection process $CD4^+CD8^-$ SP thymocytes upregulate CD25 through NF-kB activation. CD25 activation by IL-2 induce Foxp3 expression, through STAT5, establishing fully differentiated Treg cells[28,172,188–192]. In addition to IL-2, some lines of evidence suggest that IL-7, IL-15, and TGF-β signals are also involved in thymic development of Treg cells, particularly by conferring survival advantage to precursors of Tregs[28,172].

The development of tTregs is closely associated with medullary APCs in the thymus, namely, mTEC and bone-marrow-derived APCs (including DCs)[172,187,193,194]. However, it remains unknown whether each type of thymic APC is responsible for the generation of Tregs with unique TCR specificities, as mTECs and various DC subsets differ in their ability to express, capture and present antigens[172]. mTEC are also likely to present different antigens owing to the stochastic expression of tissue-specific antigens regulated by AIRE[24–26,195].

Functional Specialization of Treg Cells

Although the main cell biological aspects of Treg cells have been broadly established, defining experimentally the key mediators of Treg cell function proved surprisingly difficult. From a functional perspective, the various potential suppression mechanisms used by Treg

cells can be grouped into four categories: a) suppression by inhibitory cytokines; b) suppression by cytolysis; c) suppression by metabolic disruption; d) suppression by modulation of DC maturation and/or function[28,196,197].

The contribution of IL-10, TGF-β and IL-35 inhibitory cytokines to the function of Treg cells is still controversial. Although, Treg cells control inflammatory responses in murine models of colitis and asthma in a IL-10 dependent manner, blocking IL-10 with neutralizing antibodies or transferring IL-10-deficient Treg cells did not change the course of disease[198,199]. Importantly, while the central tenet of Treg cell function is their prevention of autoimmune responses, Treg cell-specific deletion of IL-10 did not result in the development of spontaneous systemic autoimmunity[200]. These findings suggest that Treg cell-derived IL-10 may function restrictively in the control of inflammatory responses[196,200]. TGF-β role as a mediator of Treg cell function is even more controversial. However, since the report that exosome membrane-tethered TGF-β enhanced suppressive function of Treg cells in tumour microenvironment re-surfaced the interest in this cytokine[201,202]. IL-35 was recently added to the Treg cell portfolio, as IL-35 was proven sufficient for Treg cell-mediated activity[203].

Granzyme A and B-dependent cytolysis has also been pointed out as a regulatory mechanism of Treg cells, specifically targeting B cells[164,204,205].

A long-standing proposed mechanism for Treg cell function is metabolic disruption, mainly by IL-2 depletion, a critical survival cytokine for effector cells[164,196,206]. Again, some studies questioned the role of IL-2 depletion as a *bona fide* Treg cell tool, since CD25-deficient Treg cells (which are not able to consume IL-2) kept their suppressive capacity *in vitro*[190,207]. Conversely, other studies demonstrated that Treg cells induce IL-2-deprivation mediated apoptosis[208]. Interestingly, Treg cells were also found to suppress effector cells by generating pericellular adenosine (by the action of ectoenzymes CD39 and CD73) and to transfer potent inhibitory second messenger cAMP into effector T cells[209–213].

In addition to the direct effect of Treg cells on T-cell function, Treg cells also modulate the maturation and/or function of DCs, mainly through CTLA-4-mediated mechanisms (detailed description in next sections)[155,214–217].

Overall, several models have been proposed to support a cell-intrinsic and -extrinsic function

of Treg cells. How might these multiple mechanisms be integrated and used productively by Treg cells is still unclear. It has been proposed that Treg cells use predominantly one or two crucial suppressive mechanisms (consistently in various regulatory settings), but Treg cells are able to alter their phenotype, migration and function in response to specific cues[196,197]. Additionally, Treg cells seem to be also involved in biological processes beyond suppression, namely tissue repair and anti-inflammatory protection[218].

These possible mechanisms have been discussed from the perspective of the existence of a single homogeneous Treg cell population. However, to function properly, Treg cells must modulate the activities of a wide variety of cellular components of both the innate and the adaptive systems, and this depends on their ability to come into physical proximity with their targets. Therefore, Treg cells need to operate in specific tissues and microenvironments. Thereby, Treg cells are a heterogeneous population formed by distinct cell subsets to ensure a tissue and context-specialized function[197]. Several groups demonstrated that Treg cells use canonical Th cell-associated transcription factors (namely, T-bet, IRF4 and STAT3) to maintain or restore immune homeostasis during polarized Th1, Th2 and Th17 cell-driven immune responses[197,219–222]. Furthermore, $CCR7^{low}CD62L^{low}CD44^{hi}KLRG1^{+}CD103^{+}$ tissue-resident Treg cell subsets are required to maintain immunological tolerance, namely in the skin, lung and gut[223–225].

Distinctive Features of Treg Cells in Humans

Human Treg cells were first characterized as $CD4^{+}CD25^{+}$ T cells in 2001 by several groups based on the finding in 1995 that mouse Treg cells constitutively express CD25[146,226–230]. Following the identification of Foxp3 as the gene leading to pathology in scurfy mice, Foxp3 was confirmed as a master regulator of human Treg cells[151,156,158,159]. Whereas, both murine and human Treg cells express Foxp3, Treg cells are not the unique Foxp3-expressing cell population in the human immune system. Indeed, a significant proportion of human activated T cells was found to express (transiently) Foxp3 without acquiring any regulatory activity[231–234]. Importantly, some peripheral blood non-regulatory $Foxp3^{low}$ T cells might actually produce IL-17 and IFN-γ pro-inflammatory cytokines, as shown by Miyara and colleagues[235]. Increasing the complexity of field is the fact that Treg cells are highly heterogenous in humans[171,236].

Given the known similarities between mouse and human thymocyte development, it is likely

that developmental requirements described above for mice Treg cells are similar for human Treg cell differentiation. Contrasting to murine studies, Foxp3 expression by thymocytes was observed at DP stage in humans, which concomitantly expressed ICOS, GITR and CTLA-4[237–240]. Like in mice thymus, human Treg cell development requires TCR stimulation and several signalling and molecular factors, namely, JAK3/STAT-5, Notch, CD80/CD86, ICOS/ICOSL, CD40/CD40L, TSLP, as well as cytokines, such as IL-2 and IL-15[240–244]. In humans, a unique structure in the thymic medulla, called Hassall's corpuscles, may contribute to form a TSLP-enriched niche to support Treg cell precursors[171,241,242].

Unlike murine Treg cells, human Treg cells have two distinct and clearly defined subsets: $CD45RO^{-}(CD45RA^{+})CD25^{low}Foxp3^{low}$ resting and $CD45RO^{+}(CD45RA^{-})CD25^{hi}Foxp3^{hi}$ effector Treg cells[171,235]. The existence of a resting Treg cell population in humans was reported by several groups[245–248]. Resting Treg cells have a comparable *in vitro* suppressive capacity and Foxp3-expression levels with their effector counterparts[235,245–247]. Hence, resting Treg cells are enriched for recent thymic emigrants (as they have a higher T cell receptor excision circle (TREC) content and decrease with aging) and are $Ki67^{low}$ quiescent cells[245–247]. Importantly, resting Treg cells proliferate and acquire the expression of CD45RO upon *in vitro* stimulation, suggesting that resting Treg cells represent a pool of Treg cells recently emigrated from the thymus which will convert into effector Treg cells upon peripheral activation[171,235,247]. Albeit considered naïve, CD45RA-expressing Treg cells must have undergone some TCR signalling, as peripheral maintenance of Treg cells requires continuous TCR stimulation, presumably by self-antigens[249,250]. Effector Treg cells are highly proliferative and apoptotic prone activated cells, expressing high levels of GITR, CD95 and CTLA-4[235,247]. ICOS and HLA-DR expression have been also shown to define two effector Treg cell subsets[226,251,252]. Indeed, Ito and colleagues demonstrated that $ICOS^{+}$ and $ICOS^{-}$ effector Treg cells actively produce, respectively, the inhibitory cytokines IL-10 and TGF-β *in vitro*[251]. Similarly, Baecher-Allan and colleagues claimed that HLA-DR-expressing Treg cells are a subset of terminally differentiated Treg cells with superior suppressive capacity as compared to $HLA\text{-}DR^{-}$ Treg cells[252].

The precise molecular mechanisms of suppression of human Treg cells remain to be determined, as functional studies with human Treg cells have been exclusively performed *in vitro*[171]. With this caveat, some studies have found that human Treg cells cannot suppress pro-inflammatory cytokine production and proliferation in conditions in which effector T

cells were strongly activated[171,226,253].

3.3. T Follicular Regulatory Cells

In 2011, the earlier observations showing that Foxp3-expressing Treg cells were required to control humoral responses gained a new importance following the identification of Tfr cells as a specialized cell subset by three independent groups (including our own)[254–256]. This Foxp3$^+$ T cell subset co-expressed, simultaneously, markers of Treg and Tfh cells, therefore maintaining a suppressive function and gaining ability to access B cell follicles and GCs (**Table 3**).

Table 3: Phenotypic markers of Treg, Tfr and Tfh cells in mice
Adapted from Sage *at al*[257–260]

		Treg cells	Tfr cells		Tfh cells	
			Lymph Nodes	Blood	Lymph Nodes	Blood
Regulatory markers	Foxp3	++	++	++	–	–
	Blimp-1	+	+	?	–	–
	CTLA-4	++	+++	++	+	?
	CD25	+++	++	++	–	–
	GITR	++	+++	?	–	–
Follicular markers	Bcl-6	–	+	?	++	?
	BTLA	–	++	?	++	?
	CXCR5	–	++	++	+++	++
	ICOS	+	+++	+	++	+
	PD-1	+/–	++	?	++	?

Our group found a population of Foxp3$^+$ T cells within GC of different mouse models following immunization with OVA in alum. This regulatory T cell population peaked at day 12 after immunization, when the total number of T cells within GC is already decreasing. This population shared regulatory and follicular markers with Treg and Tfh cells, respectively[256]. In parallel, Chung and colleagues found a population of CXCR5$^+$Bcl-6$^+$Foxp3$^+$ T cells in human tonsils and in mice. This population was present in organs with spontaneous GC reactions, and were also increased in draining lymph nodes upon immunization with keyhole limper hemoyanin (KLH) in complete Freund's adjuvant (CFA)[254]. Finally, Linterman and colleagues also found a population of CXCR5$^+$PD-1$^+$Foxp3$^+$ T cells following SRBC immunization that resembled activated Treg cells, but

they also co-expressed many known Tfh cell markers. Notably, Tfr cells did not express the Tfh cell molecules IL-21, IL-4 or CD40L[255].

Although, Tfr cells are now considered to be the major regulator of humoral responses to limit potential autoimmune reactions, this function might not be unique to Tfr cells. Other cell subsets, namely Qa-1-restricted $CD8^+$ T cells, might play a role in abrogating Tfh cell responses[261,262].

Since their discovery, Tfr cells have been regarded as a putative important player in the pathogenesis of human diseases characterized by disrupted GC responses, like autoimmune and chronic infectious diseases. In recent years, Tfr cells have been extensively studied. While, many aspects of Tfr cell biology were already established in mouse models, their origin, differentiation and function in humans remains elusive.

3.3.1. Biology of Tfr Cells

The first three studies describing Tfr cells reported these cells derived from thymic Treg cells[254–256]. Chung and coworkers showed that when wild-type naïve $CD4^+$ T cells and $CXCR5^-Foxp3^+$ Treg cells with different congenic markers where co-transferred into T cell-deficient ($Tcrb^{-/-}$) mice, virtually all Tfr cells were generated from Treg cells (and not naïve $CD4^+$ T cells) following KLH-CFA immunization[254]. Linterman and colleagues observed that TCR transgenic $CD25^-$ T cells, recognizing hen egg lysozyme (TCR^{HEL}), did not differentiate into Tfr cells following HEL immunization (even though they readily gave rise to Tfh cells)[255]. Additionally, Tfr cells were shown to derive only from thymic $Foxp3^+CD4^{SP}$ T cells (but not $Foxp3^-CD4^{SP}$ T cells)[255]. Our group used another model of T cell-deficient ($Tcra^{-/-}$) mice as recipient of wild-type Tfh and thymic Treg cells to demonstrate that only the $PD\text{-}1^-CXCR5^-Foxp3^{GFP+}$ thymic Treg cells could differentiate into Tfr cells[256].

Recently, Aloulou and colleagues used MHC class II tetramers to demonstrate the existence of a small population of antigen-specific Tfr cells. This population emerged following myelin oligodendrocyte (MOG) in CFA immunization of $Mog^{-/-}$ mice (where MOG acts like a foreign antigen) and wild-type mice (although less extensively)[263]. Moreover, in $CXCR5^{-/-}$ bone marrow chimeras, where all T cells lack CXCR5, antigen-specific Tfr cells originated from transferred naïve $CD4^+$ T cells (sorted from Foxp3-depleted mice), showing that Tfr

cells can arise from peripherally-induced Treg cells[263]. While, these interesting findings suggest that the origin and TCR specificity of Tfr cells may be different in non-self and self-induced immune responses, recent work from our group highly supports the origin of Tfr cells from thymic Foxp3$^+$ Treg cells[264]. Using adoptive cell transfer models, we observed that transferred OVA-specific TCR-transgenic CD4$^+$ T cells from Rag$^{-/-}$ mice (devoid of Foxp3$^+$ Treg cells) into Rag-sufficient mice did not give rise to Tfr cells following OVA-IFA immunization. These results confirmed previous reports, and further validated Tfr cell origin from Foxp3$^+$ thymic Treg cells under physiological conditions, as lymphopenia in Rag$^{-/-}$ hosts (where original experiments were performed) induce T cell homeostatic proliferation and expansion[265,266]. Importantly, we found that recruitment of Tfr cells into GC was independent of the immunizing antigen. Indeed, while most Tfh cells found within GC of OVA-immunized C57BL/6 mice derived from OVA-specific TCR-transgenic CD4$^+$ T cells, no OVA-specific Tfh cells were found within GC of β-lactoglobulin-immunized mice. On the contrary, virtually no OVA-specific Tfr cells were found within GC of mice in the two immunizing conditions (OVA-specific TCR-transgenic Foxp3$^+$ T cells specifically recognized and proliferated in the presence of OVA)[264]. The lack of specificity of Tfr cells towards the immunizing non-self-antigen was further observed by the lack of antigen-specific MHC class II tetramer-positive cells upon immunization with the corresponding antigen. Indeed, we could not detect antigen-specific tetramer-positive Tfr cells in any of the conditions tested[264]. Also, Tfr cells did not preferentially proliferate or survive *in vitro* with immunizing antigen signals when compared with a control antigen. Therefore, it is reasonable to assume that activation and recruitment of Tfh and Tfr cells into GC is governed by different signals. To further address specificity of Tfr cells, we sequenced the TCRα-chain (*TRA* gene) of Tfr, Tfh, and Treg cell populations from immunized TCRβ-restricted mice (with the cells sorted at the peak of GC reaction). The TCR repertoire analysis showed that, although Tfr cells are an oligoclonal population, they have a TCR repertoire that resembles the repertoire of Treg cells, but that is different from that of Tfh cells[264]. The oligoclonal expansion of Tfr cells (which is consistent with their generation only following immune adaptive reactions) provides evidence for the existence of a driving (self) antigen for Tfr cell differentiation. Sage and colleagues consistently demonstrated that Tfr cells sorted from mice immunized with different 4-hydroxy-3-nitrophenylacetyl hapten (NP) conjugated antigens (NP-HEL and NP-OVA) equally suppressed B and Tfh cells sorted from NP-OVA in CFA immunized mice[258]. Although, these experiments were performed *in*

vitro, they indicate that Tfr cells do not require specificity to the immunizing antigen for their suppressive capacity.

While, the differentiation of Tfr cells is still not as characterized as the differentiation of Tfh cells, Tfr cells seem to undergo a multistep Bcl-6-dependent differentiation process like Tfh cells.

For Tfh cells, such complex process starts during T cell priming by DCs and continues throughout T-B interactions until Tfh cells differentiate into mature GC Tfh cells (as described above)[63–65,115]. Like other naïve $CD4^+$ T cells, Tfr cell differentiation is initiated at the time of T-cell priming by DCs, as diphtheria toxin-induced depletion of $Foxp3^{DTR}$ Treg cells at the time of immunization results in the lack of Tfr cells, despite the recovery of Treg cells and normal GC formation[255,267]. Sage and colleagues established the DC requirement for Tfr cell differentiation by depleting DCs with diphtheria toxin in CD11c-DTR bone-marrow chimeric mice[258]. In these experimental settings, transfer of NP-OVA-pulsed LPS-activated DCs led to expansion of Tfr cells (as well as Tfh cells) in the draining lymph node of recipient mice. Additionally, antigen presentation by DCs (and not only survival and co-stimulatory signals) was required for Tfr cell differentiation, as $Ciita^{-/-}$ DCs (which are unable to present antigens) were impaired in their ability to induce Tfr cell differentiation[258,268]. However, the DC subsets most directly responsible for stimulating Tfr cell differentiation remain unclear.

The full differentiation into GC Tfr cells is also dependent on B-cell interactions. When mice lacking B cells (μMT mice) were immunized with NP-OVA or SRBC a significant reduction in lymph node Tfr cells were observed[258,255]. Moreover, Linterman and colleagues showed SAP-deficient $Sh2d1a^{-/-}$ mice have virtually no Tfr cells following SRBC immunization, clearly showing B-cell dependency of Tfr cells, similarly to Tfh cells[89,255]. Notwithstanding, B-cell requirement for Tfr cell differentiation is controversial. Indeed, B cells were only required for development of full differentiated Tfr cell differentiation, as putative Tfr cells were found in blood of μMT mice following immunization[258]. This population of circulating Tfr cells had a lower expression of ICOS and a less suppressive capacity, when compared to Tfr cells sorted from lymph nodes. Although some Tfr cells were found in blood of unimmunized mice, Tfr cells were shown to originate upon first contact with DCs and before full commitment to the GC fate. The same study also showed that circulating Tfr cells can

persist in the system for long periods of time and be later recruited into the GC to suppress further responses[258]. These studies suggest that Tfr cell effector activity is initiated during contact with DCs in the T cell zone, strengthened in the inter-follicular region during contact with B cells, and optimized in the GC (**Figure 1**).

Another similarity between the differentiation processes of Tfr and Tfh cells is the requirement of TCR stimulation and co-stimulatory signals through CD28 and ICOS[63–65,70,73,115]. The CD28 requirement for Tfr cells commitment was first shown with bone-marrow chimeras, with mixed CD28-deficient and -sufficient cells, under which conditions both Tfr and Tfh cells originated exclusively from CD28-sufficient cells[255]. These results were later corroborated by a study showing the absence of Tfr and Tfh cells in $CD28^{-/-}$ and in $Icos^{-/-}$ mice[260].

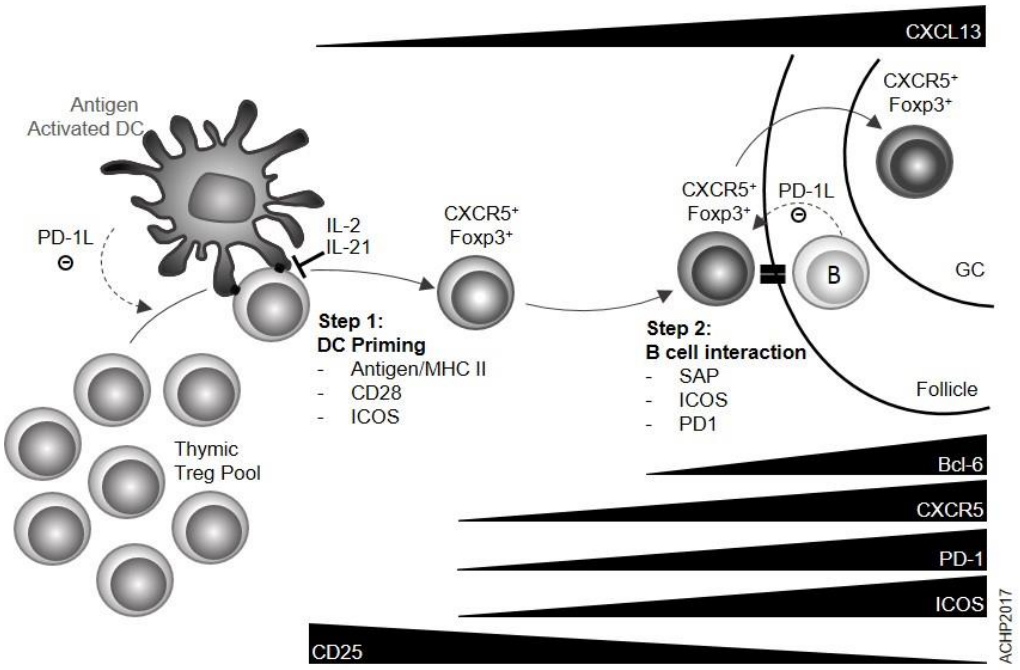


Figure 1: Tfr cell differentiation signals and pathway.
Tfr cells originate from thymic Treg cells upon DC priming (from activated DCs). This process involves antigen / MHC II signals, as well as CD28 and ICOS signalling. This first step of Tfr cell differentiation is inhibited by IL-21, IL-2, and PD-1L. After this process Tfr cells upregulate Bcl-6, CXCR5, PD-1 and ICOS. CXCR5 will guide the cells toward CXCL13 enriched B-cell zones (follicles and GC). At the T-B border, Tfr cells receive full differentiation signals from activated B cells (it is not known whether these are cognate B cells) in a SAP, ICOS and PD-1-dependend manner. PD-1L is also an inhibitory signal at this stage. Throughout this process CD25 expression decreases.

There are, however, some differences between the differentiation processes of Tfr and Tfh cells. A striking difference is that only the differentiation of Tfr cells seems to be affected by co-inhibitory signals (**Figure 1**). PD-1 signalling exclusively inhibit Tfr cell differentiation, as Pdcd1$^{-/-}$ (PD-1 deficient) mice had higher frequencies of Tfr cells both in lymph nodes and blood (but normal frequencies of Tfh cells) following MOG-CFA immunization[260]. CXCR5^{-}Foxp3^{+} Treg cells transferred from Pdcd1$^{-/-}$ mice showed a greater capacity for CXCR5 upregulation, suggesting that PD-1 specifically controls Tfr cell differentiation from thymic Treg cells[260]. The direct impact of PD-1 in Tfr cell differentiation was also corroborated by the lower proliferating rate (analysed by Ki-67 expression) of Pdcd1$^{-/-}$ Tfr cells. A lower proliferative rate excluded increased cell maintenance as the mechanism for Tfr cell expansion in Pdcd1$^{-/-}$ mice. PD-1 represses Tfr cell differentiation prior to B-cell interaction, as blood Tfr cells were also increased in PD-1 deficient mice[260]. Importantly, in elegant *in vitro* assays, Sage and colleagues showed a greater suppressive capacity of Pdcd1$^{-/-}$ Tfr cells (measured by proliferation of Tfh responder cells and supernatant IgG1 concentration)[260]. PD-L1 (expressed by all B cell and DC subsets) mediates the role of PD-1 signalling in Tfr cell differentiation, since only PD-L1-deficient mice (but not PD-L2-deficient mice) could replicate the Tfr phenotype observed in PD-1-deficient mice[260].

Another inhibitory molecule that has a negative impact on Tfr cell differentiation is CTLA-4. Two groups showed that Tfr cells expressed higher amounts of CTLA-4 in comparison to total Treg population, thus CTLA-4 was proposed as a universal marker of functionally competent Tfr cells[259,269]. However, CTLA-4 expression on Tfr cells was identical to CTLA-4 expression on activated CD44^{+} Treg cells, suggesting that the enhanced CTLA-4 expression by Tfr cells might be a result of their activated state rather than an intrinsic difference from non-follicular Treg cells[269]. These two groups demonstrated that deletion of CTLA-4 led to an increased frequency and absolute numbers of Tfr cells[259,269]. Sage and colleagues showed an expansion of Tfr cells in lymph nodes and blood by CTLA-4 depletion induced by tamoxifen administration to UBC-ERT-iCre^{+}Ctla4$^{F/F}$ mice three days before immunization with NP-OVA in CFA[259]. Using this system, these authors circumvented the effects of germline deletion of CTLA-4 known to induce spontaneous development of systemic lymphoproliferation, fatal T-cell-mediated autoimmunity and hyperproduction of immunoglobulin E[214]. Conditional deletion of CTLA-4 in Treg cells (using tamoxifen administration in Ctla4 floxed mice bred to Foxp3-ERT2-Cre-GFP knockin mice)

recapitulated the impact of global CTLA-4 deletion both *in vitro* and *in vivo*[259]. Wing and colleagues used CTLA-4$^{flox/flox}$, CTLA-4$^{flox/wt}$ and CTLA-4$^{wt/wt}$ Foxp3$^{IRES\text{-}Cre+}$ hemizygous male mice to demonstrate an expansion of Tfr cells as well as greatly increased spontaneous GC formation in the complete absence of CTLA-4 in Treg cells (as CTLA-4$^{flox/wt}$ showed normal levels of Tfr cells)[269]. Although, CTLA-4 depletion greatly impacts Tfr cell population, whether this is due to a direct effect on differentiation or on cell maintenance is still not clear.

The cytokine IL-21 also has a negative impact on Tfr cell numbers even though it supports Tfh differentiation (with IL-6 redundancy) (**Figure 1**)[61,81,270–272]. Jandl and colleagues studied follicular T cells and humoral responses in Il21r$^{-/-}$ mice. Ki-67^{+} proliferating Tfr cells specifically expanded following SRBC immunization (no expansion of Tfh cells was observed) in IL-21 receptor-deficient mice. To prove a Tfr cell-intrinsic role of IL-21 receptor, Jandl and colleagues used 1:1 mixed bone marrow chimeras with Il21$^{-/-}$ Thy1.2 T cells and WT Thy1.1 T cells. IL-21 receptor-deficient T cells preferentially differentiate into Tfr cells following SRBC immunization[270]. Interestingly, CD25 expression levels on IL-21 receptor-deficient Tfr cells approximated that of their Treg cell precursors, leading to a heightened signalling in response to IL-2 (as Il21$^{-/-}$ Tfr cells greatly expanded after Il-2:IL-2mAb complexes administration and increased STAT5 phosphorylation after recombinant IL-2 antibody treatment)[270]. Using different *in vitro* approaches, Jandl and colleagues showed that Bcl-6-deficient CD4^{+} T cells had an increased CD25 expression, and an increased frequency of CD25^{hi} T cells upon IL-21 neutralization[270]. Therefore, they proposed that IL-21 induces Bcl-6 expression which in turn limits CD25, the reduction of CD25 expression then leads to lower responsiveness to IL-2, consequently IL-21 restrains Tfr cell expansion by limiting CD25^{+} Tfr cell proliferation. Consistently, Ding and colleagues revealed that IL-21 can suppress Tfr cell differentiation in BXD2 autoimmune mice[273]. Tfr cells were found to be increased in Il21$^{-/-}$ BXD2 mice, in parallel with a reduction in GC B cells and GC size in that murine model[273]. In *in vitro* systems, they found that IL-21 induced STAT3 phosphorylation and decreased Foxp3 expression, by a Akt-dependent mechanism[273]. Whether the IL-21-induced decrease in Foxp3 expression by already formed Tfr cells is sufficient to reduce Tfr cell numbers *in vivo* is not known. Nevertheless, as IL-21 is mainly secreted by Tfh cells and orchestrate several components of humoral responses, Tfr cell differentiation inhibition by IL-21 evoke a feedback control mechanism in the GC response[82,274]. Another study also supports IL-21 as a repressor

cytokine for Tfr cells. Sage and colleagues found that IL-21 diminished Tfr cells proliferation (based on proportion of Ki-67$^+$ Tfr cells) in cocultures with Tfh and B cells[272].

The work by Jandl and colleagues showed that Tfr cells comprise cells that express CD25 and cells that lack CD25[270]. Although, CD25$^-$ Tfr cells express higher levels of Bcl-6, proliferation of CD25$^+$ Tfr cells was responsible for the expansion of Tfr cells in IL-21 receptor deficient T cells, where the lack of IL-21 receptor signalling limit Bcl-6 expression[270]. How Tfr cells cope with IL-2/CD25 signalling (critical for Treg cell development and maintenance) and Bcl-6-dependent T follicular program (which is repressed by IL-2/CD25 signalling) has been regarded as one of the most intriguing features of Tfr cells[28,83–85,87,223,275]. Recently, three independent groups confirmed that Tfr cells have low CD25 expression[276–278]. Wing and colleagues showed that both CD25$^-$ and CD25$^+$ Tfr cells differentiated and expanded upon NP-OVA in alum immunization. However, while CD25$^+$ Tfr cells persist after the peak of GC reaction, CD25$^-$ Tfr cells followed a kinetic response similar to Tfh cells (albeit with a later peak), suggesting that only CD25$^-$ Tfr cells followed a GC-like kinetic response[276]. Moreover, CD25$^-$ Tfr cells more closely resembled Tfh cells when their gene profile was analysed and preferentially localize within GC[276]. Functionally, CD25$^-$ and CD25$^+$ Tfr cells had similar suppressive capacity, as both subsets suppressed IgG1 production by B cells *in vitro*[276]. To specifically address the impact of IL-2 on Tfr cells, Ritvo and colleagues, used Foxp3^{GFP} reporter mice infected with adeno-associated virus coding for IL-2 (to maintain continuous high levels of IL-2) prior to OVA immunization[277]. The frequency of Tfr cells was decreased in the presence of IL-2, suggesting a negative impact of IL-2 on Tfr cells[277]. CXCR5^{hi}PD-1^{hi}GFP$^+$ Tfr cells expressed Bcl-6 and lack CD25 expression, suggesting that throughout Tfr cell differentiation Bcl-6 is progressively upregulated while CD25 is downregulated[277]. Thus, Ritvo and colleagues claimed that all Tfr cells lack CD25 expression, but they did not provide an explanation for the existence of CXCR5$^+$CD25$^+$ Treg cells. Interestingly, using the same CXCR5^{hi}PD-1^{hi}GFP$^+$ strategy to identify Tfr cells, Linterman and colleagues found that Tfr cells express CD25 (although at lower levels compared to conventional Treg cells) in their pioneering work[255]. Although, Tfr cells clustered with Tfh cells when their transcriptomic profile were analysed, Ritvo and colleagues found that Tfr cells preferentially expand when insulin (a self-antigen) was used as the immunizing antigen[277]. These findings are consistent with a similar TCR repertoire between Tfr and Treg cells[264]. Thereby, Tfr cells acquire a Bcl-6-depedent transcriptome throughout their differentiation process but

retain a thymic-derived Treg cell self-biased TCR repertoire. $CD25^+$ Tfr and $CD25^-$ Tfr cells were also found in PR8 influenza virus infected mice[278]. However, while previous studies have shown that Tfr cells quickly develop following soluble protein immunization, under physiological conditions (influenza infection further validated with LCMV infection), Tfr cells only accumulated during the late phase of the primary response (day 30–60)[260,254,255,278]. Botta and colleagues found that most Bcl-$6^+$$CXCR5^+$ Tfr cells were $CD25^{lo}$ and when sorted as such they had a very distinct gene profile compared to conventional Treg cells[278]. Importantly, they consistently demonstrated that Tfr cells were derived from $CD25^+$ Treg cells, as virtually no progeny derived from $CD25^{lo}Foxp3^+$ T cells (adoptively transferred with equivalent numbers of $CD25^{hi}Foxp3^+$ T cells into $Tcrb^{-/-}$ T cell-deficient mice) upregulated Bcl-6 and CXCR5[278]. The progeny of $CD25^{hi}Foxp3^+$ T cells which differentiate into Tfr cells concurrently downregulated CD25 (**Figure 1**)[278]. In a similar system, they also confirmed that $Foxp3^-$ T cells cannot differentiate into Tfr cells following influenza infection[278]. To mechanistically address the impact of IL-2 in Tfr cell differentiation, Botta and colleagues treated influenza infected mice with recombinant IL-2. Under IL-2 treatment Tfr cells failed to develop in a Blimp-1 dependent manner[278]. They generated mixed bone marrow chimeras using WT and $Prdm1^{flox/flox}Lck^{cre}$ (Blimp-1-deficient) donors to demonstrate that Tfr cells quickly developed from Blimp-1-deficient cells as early as 10 days following influenza infection even in the presence of recombinant IL-2, suggesting that Blimp-1 limits Tfr cell differentiation and mediates the negative impact of IL-2 on this population[278]. Given that IL-2 was shown to inhibit Bcl-6 expression by upregulating Blimp-1 in Tfh cells, it is tempting to assume that IL-2 enriched conditions also block Tfr cell differentiation through a Blimp-1-dependent mechanism (**Figure 2**)[279]. Although, IL-2 seems to inhibit Tfr cell differentiation, the absence of IL-2/STAT5 signalling may lead to Foxp3 downregulation[28]. Therefore, the maintenance of Foxp3-expressing Treg cells in the absence of IL-2/CD25 (IL-2Rα) must be accomplished by other homeostatic mechanisms. Indeed, Tfr cells express high amounts of intermediate-affinity IL-2 receptor (CD122/IL-2Rβ) which may be sufficient to prevent Foxp3 downregulation[278]. Interestingly, some tissue resident Treg cells do not require IL-2 for their homeostatic control (and downregulate CD25) but instead rely on continued signalling through ICOS[280].

At the transcriptomic level, there are also similarities and differences in Tfr and Tfh cell differentiation (**Figure 2**).

As referred above, Bcl-6 is the master regulator for both subsets[58–60,256,254,255]. Two of the original groups describing Tfr cells demonstrated BCl-6 requirement for Tfr cell generation, using different approaches with Bcl-6-deficient mice[254,255]. Linterman and colleagues used mixed $Bcl6^{+/+}$:$Bcl6^{-/-}$ bone marrow chimeras to prove that cells lacking Bcl-6 expression did not give to Tfr cells following SRBC immunization (in these mice normal GC formation was observed)[255]. On the other hand, Bcl-6 was also shown to be essential for Tfr cell generation, as $Bcl6^{-/-}$ mice had a significant reduction in Tfr cells following immunization with KLH in CFA[254]. A key difference on the transcription factors present in Tfr and Tfh cells is the balance between Bcl-6 and Blimp-1. While Bcl-6-deficiency resulted in a virtually complete absence of Tfr cells, Blimp-1-deficiency increased Tfr cells, suggesting Blimp-1 limits the size of Tfr cell population[255,281]. This intricate balance seems to be required to position Tfr cells between the phenotype of Tfh and Treg cells.

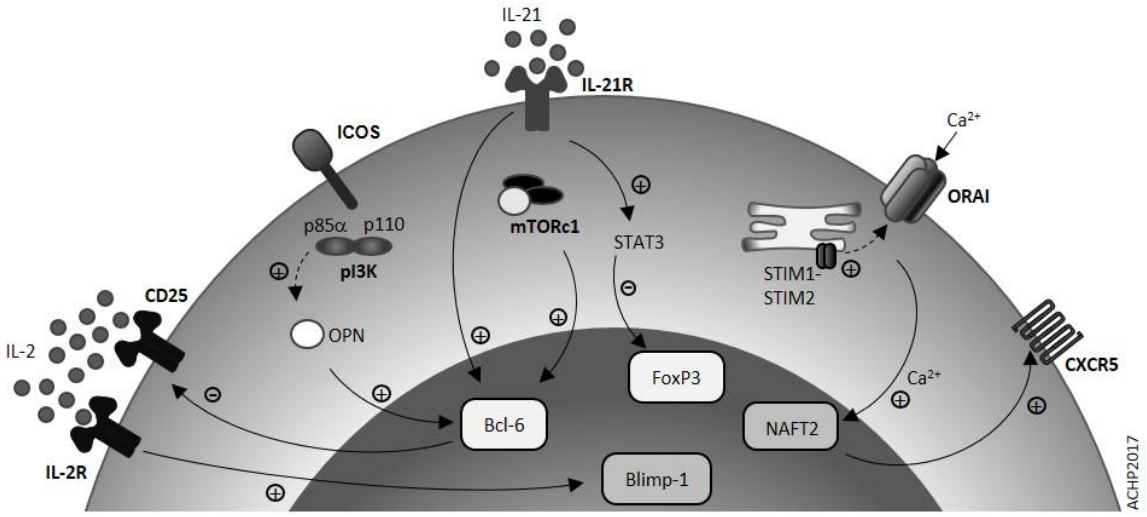


Figure 2: Signalling and transcriptomics of Tfr cells.
While, a balance between Bcl-6 and Blimp-1 is critical for the establishment of full transcriptional profile $Foxp3^+$ Tfr cells, store-operated Ca^{2+} entry (by ORAI and STIM1 and STIM2) are required for upregulation of chemokine receptor CXCR5, via NFAT2. IL-21 (secreted by Tfh cell) inhibit Tfr cell differentiation through IL-21R / STAT3 signalling, maintaining Tfh and Tfr cells regulated by a IL-21-dependent feedback mechanism. IL-2/STAT5 signalling inhibits Bcl-6-dependent Tfr cell program through Blimp-1 upregulation, therefore, environment IL-2 consumption is required to promote Tfr cell development. At the same time Bcl-6 upregulation also decreases CD25 (IL-2Rα) enforcing Tfr cell program. Additionally, ICOS signalling promotes interaction between p85α and intracellular osteopontin (OPN), followed by translocation of OPN to the nucleus, where OPN protects Bcl-6 from ubiquitin-dependent proteasome degradation. Signalling via mTOC complex 1 (mTORc1), in a STAT3-dependent manner, is also crucial for integration of diverse signals promoting Tfr cell differentiation.

Initial CXCR5 expression on Tfh cells requires Ascl2 exppression[98]. So far, Ascl2 expression on Tfr cells was not reported. On the contrary, CXCR5 expression in Tfr cells requires the expression of a Ca^{2+}-responsive molecule, called transcription factor nuclear factor of activated T cells 2 (NFAT2, also known as NFATc1)[282]. Indeed, conditional knockout mice for NFAT2 in $CD4^+$ T cells have a specific reduction of Tfr cells followed by an overall amplification of GC reactions and increased frequencies of Tfh cells upon different immunizing antigens (namely NP-KLH, OVA and SRBC)[282]. Taking advantage of adoptive cell transfer experiments (in which WT and NFAT2-deficient $CD4^+$ T cells were co-transferred to $Rag^{-/-}$ mice) and $Naft2^{flox/flox}$ mice crossed to $Foxp3^{IRES\text{-}Cre}$ mice, the effect of NFAT2 was found to be Tfr cell-intrinsic[282]. Mechanistically, NFTA2 directly binds to Cxcr5 promoter, inducing Tfr cell differentiation from thymic Treg cells by upregulating CXCR5[282].

Moreover, stromal interaction molecule 1 (STIM1) and STIM2, which mediate the activation of membrane Ca^{2+} channels on T cells upon TCR stimulation (a pathway known as store-operated Ca^{2+} entry, or SOCE), leading to activation of transcription factors of the NFAT family, were also found to be important for Tfr cell differentiation[283,284]. In conditional double knockout mice of STIM1 and STIM2 in $CD4^+$ T cells ($Stim1^{flox/flox}Stim2^{flox/flox}$ mice crossed to $Cd4^{Cre}$ mice), Tfr cells were significantly reduced. However, it is noteworthy that the Tfr cell population, even in unimmunized WT mice, was much larger than originally reported[258,256,254,255,283]. Using Rag-deficient mice reconstituted by 1:1 mixed bone marrow chimeras of WT and $Stim1^{flox/flox}Stim2^{flox/flox}Cd4^{Cre}$, Vaeth and colleagues proposed an intrinsic requirement for SOCE in Tfr cell differentiation[283]. STIM1 and STIM2-deficient Treg cells (sorted from $Stim1^{flox/flox}Stim2^{flox/flox}$FoxP3-$YFP^{Cre}$ hemizygous female mice) showed an impaired expression of Bcl-6[283].

Tumor necrosis factor receptor (TNFR)-associated factor 3 (TRAF3), and signal transducer and activator 3 (STAT3) were also found to play a role in Tfr cell differentiation[285,286]. When Chang and colleagues investigated the role of TRAF3 in homeostasis and function of Treg cells, using $Traf3^{flox/flox}$ mice crossed to $Foxp3^{Cre}$ mice, they found an amplified GC formation along with absence of Tfr cell expansion (but normal levels of Tfh cells) following SRBC immunization[286]. These findings suggested TRAF3 is critical for Tfr cell induction upon antigen stimulation. This signal adaptor has a broad spectrum of biological functions which are cell type-specific. TRAF3 binds to TCR/CD28 complex and to many other

receptors expressed by Treg cells, namely TNFR2 and GITR[287]. This provides evidence for the potential dependency of Tfr cell differentiation from inflammatory signals, such as TNF. Similarly, Wu and colleagues used $Stat3^{flox/flox}$ mice crossed to $Cd4^{Cre}$ to establish that STAT3 was necessary for Tfr cells, as Tfr cells were almost absent in those mice following SRBC immunization[285].

The p85α-osteopontin axis was also found to be required for Tfr cell differentiation, providing a link between ICOS-induced PI3K signalling and Bcl-6 upregulation in this cell population[288]. Using adoptive cell transfers, Leavenworth and colleagues demonstrated that TCR-deficient mice transferred with WT naïve T cells and osteopontin (OPN)-deficient Treg cells had substantial fewer $CXCR5^{+}PD\text{-}1^{+}CD25^{hi}$ Tfr cells (which was associated with expanded GC B cells and higher anti-KLH antibody titres) following immunization with KLH in CFA[288]. Further mechanistic studies allowed these authors to propose that upon ICOS stimulation intracellular OPN interacts with p85α (a component of PI3K complex), which promotes nuclear translocation of OPN. In the nucleus, OPN stabilizes Bcl-6 expression through interference with the ubiquitin-mediated degradation of Bcl-6[288].

Recently, Xu and colleagues addressed the role of mTOR signalling in Tfr cell fate decisions[289]. The mTOR signalling (through two functionally distinct complexes mTORC1 and mTORC2) controls various cellular processes, including cell growth, proliferation, and survival, by sensing and integrating environmental cues, taking part in T cell fate decisions[290]. Specifically, the IL-2/mTORC1 signalling axis appears to inhibit Tfh and Treg cell differentiation (although mTORC1 is important for the suppressor function of Treg cells), whereas mTORC2 was reported to be essential for Tfh cell differentiation[182,291–293]. Upon OVA-CFA immunization, Tfr cells expressed the highest amounts of phosphorylated S6 and 4E-BP1 (the key mTORC1 targets), compared to Tfh and $CXCR5^{-}$ Treg cells, suggesting a differentiating role of mTORC1 in Tfr cells[289]. Importantly, mTORC1 signalling induced *de novo* Tfr cell differentiation from thymic-derived Treg cells, as rapamycin (a selective inhibitor of mTORC1 pathway) treated conventional Treg cells showed a reduction conversion to Tfr cells post OVA-CFA immunization, when adoptively transferred into WT recipient mice[289]. Consistently, $Rptor^{flox/+}Foxp3^{Cre}$ mice (which harbour a selective but partial reduction of mTORC1 signalling in Treg cells to avoid the impact of complete loss of mTORC1 signalling on their homeostatic proliferation) had a reduced proportion and absolute numbers of Tfr cells after NP-OVA in CFA immunization[289].

Moreover, mTORC1 signalling is also important for Tfr suppressive capacity, as when rapamycin-treated Tfr cells formed upon LCMV infection were adoptively transferred with WT Tfh cells (also sorted from LCMV infected mice) into SAP-deficient mice (which fail to generate functional Tfh and Tfr cells upon infection), they failed to regulate GC reactions. Indeed, SAP-deficient mice transferred with rapamycin-treated Tfr cells showed an substantial expansion of GC B and plasma cells, indicating that mTORC1 signalling is required for differentiation of Tfr cells, as well as for their suppressive functions upon infection[289]. The gene set enrichment analysis of gene signatures in WT and $Rptor^{-/-}$ Tfr cells (sorted from $Raptor^{flox/flox}CD4^{Cre}$ mice after OVA-CFA immunization) implied mTORC1 signalling played an important role in many features of Tfr cell lineage, as the Tfr cell gene signature was substantially reduced in mTORC1-deficient Tfr cells[289]. Using a sequential set of vector-transduced $Rptor^{-/-}$ Treg cell experiments, Xu and colleagues also proposed that mTORC1 signalling mediates Tfr cells conversion from conventional Tregs through a STAT3-TCF-1-dependent Bcl-6 upregulation[289].

Finally, members of the helix-loop-helix family (E and Id proteins) also contribute to Tfr cell fate decision[294,295]. Miyazaki and colleagues observed a spontaneous Th2 cell-mediated fatal inflammation in mice lacking Id2 and Id3 expression in Treg cells. In these $Id2^{flox/flox}Id3^{flox/flox}Foxp3^{Cre}$ mice spontaneous formation of IgG1 and IgE producing GC and expansion of IL-4, IL-5 and IL-13-secreting Tfh cells was associated with an increased proportion of $CXCR5^{+}PD\text{-}1^{+}$ Treg cells in lymphoid organs[294]. Despite expression of some follicular markers, this cell population did not fully differentiate into Bcl-6^{+} Tfr cells. As upon *in vitro* exposure to TCR-mediated signalling Treg cells downregulated Id3 and E2A-bound sites were found to be associated with putative enhancer regions across the Cxcr5 loci, the authors proposed that upon TCR-mediated signalling, Id2 and Id3 levels decline permitting E-protein binding activity and induction of a Tfr cell prone transcription signature. Later on, Id2 and Id3 abundance is required to modulate Bcl-6 and Blimp-1, ultimately leading to the development o
f a mature Tfr cell population[294]. Thus, it is likely that a default Tfr-cell program is induced in Treg cells upon TCR-mediated activation, leading to full differentiated Tfr cells whenever additional signals are present, namely B-cell interactions and (probably) low exposure to IL-2.

Although, the same type of cellular interactions seems to trigger both Tfr and Tfh cells differentiation, kinetic studies of the accumulation of both populations after immunization revealed that Tfr cells appear in the GC at later time points reaching their maximum frequency around 5 days later than Tfh cells do[260,256,277,278,296,297]. This unsynchronized accumulation of Tfr and Tfh cells may be required for the initial establishment of the GC, where few Tfh and GC B cells need to rapidly expand without the suppressive action of Tfr cells. On the contrary, once the GC has reached a significant size, Tfr cells would be important for the control of the GC reaction and its outcome. Despite their accumulation upon antigen stimulation, the individual contribution of Tfr cells to the total $CXCR5^+$ T cell population decrease with time, suggesting a disproportionate expansion of Tfr and Tfh cells during adaptive immune responses[297].

3.3.2. Mechanisms of Tfr Cell Function

Tfr cells constitute a regulatory T cell subset specialized in the regulation of the GC response. By directly modulating Tfh cell proliferation, B cell metabolism and cytokines secreted by Tfh cells in secondary lymphoid organs, Tfr cells modify GC outcomes at several levels: a) control of GC size; b) selection of antigen specific Tfh and B cell clones; and c) modulation of class switch and affinity maturation of antibodies (**Figure 3**).

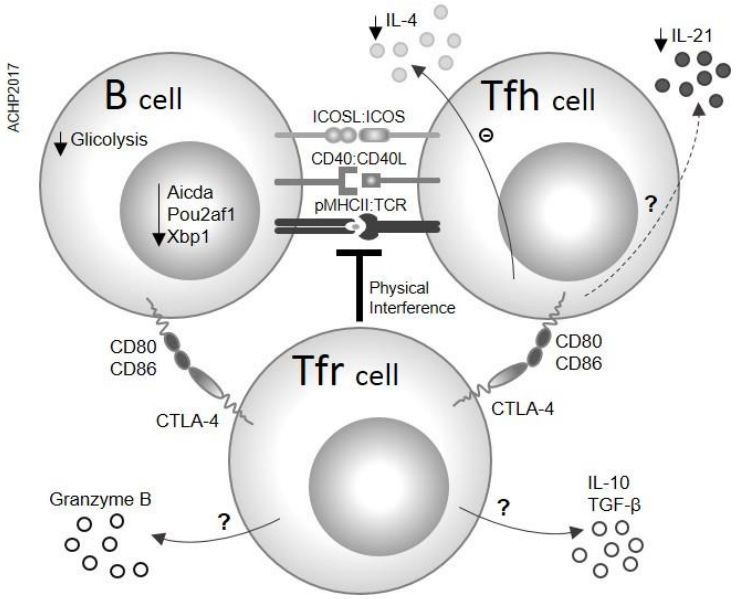


Figure 3: Mechanisms of Tfr cell-mediated regulation of humoral responses.
Tfr cells regulate T – B interactions within GC mainly by physical interference at the immunological synapse, which is required for the survival feedback loop between GC B cells and Tfh cells. CTLA-4 is a key molecule of Tfr cell function at immunological synapse, as it directly blocks CD80/CD86 co-stimulatory signals. Using these mechanisms, Tfr cells impair GC B cell

metabolism (mainly by decreasing glucose uptake and usage) and induce the downregulation of GC B cell effector molecules, such as Pou2af1 (required for GC B cell formation), Xbp1 (required for antibody secretion) and Aicda (required for class switch recombination). On the other side of the immunological synapse, Tfr cells limit IL-21 and IL-4 secretion by Tfh cells capacity is also modulated y the presence of Tfr cells. Granzyme B, IL-10 and TGF-β secretion by Tfr cells may also account for their regulatory capacity.

The precise molecules that underpin such effects are largely unknow, but undoubtedly encompass CTLA-4-mediated suppression (see below). Hypothetically, other molecular mechanisms may be involved in Tfr cell function, namely secretion of IL-10 and TGFβ, induction of cell death by granzyme-mediated apoptosis, and cell contact by unknow mechanisms (**Figure 3**).

Control of GC Outcomes

The initial studies ascribed control of GC size and of immunoglobulin class and antibody affinity to Tfr cell population[256,254,255]. Indeed, Tfr cells are critical to control GC size and immunoglobulin production, as Tcrα$^{-/-}$ mice transferred with OT-2 cells (from mice without Treg cells) plus CXCR5-deficient Treg cells had greater GC volumes and higher serum titres of OVA-specific IgM and IgG2a, following OVA-Alum immunization[256]. Using a similar adoptive cell transfer experiment, Chung and colleagues showed that mice lacking either CXCR5 or Bcl-6-expressing Treg cells had expanded GC B cells and a higher proportion of NP-specific GC B cells following NP-KLH-CFA immunization. Moreover, in the absence of Tfr cells higher amounts of high-affinity NP-KLH-specific IgG1 and IgG2 antibodies were observed[254]. However, the impact of Tfr cells in selection of antigen specific cells and antibody affinity maturation is controversial. In mixed bone marrow chimeras (1:1 ratio of Sh2d1a$^{-/-}$Foxp3^{DTR} or Sh2d1a$^{+/+}$Foxp3^{DTR} cells), Linterman and colleagues observed an increase in Tfh and GC B cells, but a reduced proportion of NP-specific GC B cells (but no differences in antigen-specific antibodies) when mice lacking Tfr cells were immunized with NP-KLH in alum[255]. Curiously, in secondary immune responses (NP-KLH boost at day 24 after primary immunization), high-affinity NP-specific antibody titres were lower in Treg and Tfr cell depleted mice, suggesting that kinetics and modulation of GC reaction by Tfr cells is different in primary and secondary immune responses[255]. These reported differences in GC outcomes may be, at least partially, assigned to the nature of adjuvants used[298–300]. Curiously, in murine models of influenza vaccination (where adjuvants are not used and antigens are not proteins) virtually no Bcl-6^{+} Tfr cells were generated at the peak of GC reaction (days 7 – 15), albeit Tfr cell were detected at later time points (day 30)[278,301].

Additionally, the methods used to induce Treg cell depletion might also explain these conflicting results, as excessive IL-2 signalling after Treg depletion was shown to restrain antigen-specific Tfh cell responses[206,301].

Whereas these pioneer reports showed contradictory results in the absence of Tfr cells, more recent studies support the concept that Tfr cells restrain generation of antibodies specific to the immunizing antigen while favour the emergence of B cell clones secreting high affinity antibodies[259,269,278,302,303]. In a recent study, an increased percentage of NP-specific GC B cells and NP-specific IgG2a and IgE titres was observed when Treg cells were not allowed to generate Tfr cells. By diphtheria toxin administration, these $Foxp3^{DTR}$ mice showed a persistent Tfr cell depletion at the peak of GC response in parallel with Treg cell recovery[269]. Interestingly, Wing and colleagues proposed that while antigen-specific Treg cells might suppress the relevant response, polyclonal Treg cells are critical for the maintenance of immune homeostasis. Indeed, upon transient Treg cell depletion on the time of immunization an increased proportion of antigen-specific Tfh cells was observed, while an overall reduction of antigen-specific GC B cells (as well as increased anti-dsDNA autoantibody levels) was noticed when Treg cells were sustainably depleted[269]. Sage and colleagues showed that when $Cd28^{-/-}$ mice (which cannot generate Tfh and Tfr cells) were transferred with a pool of $CXCR5^{+}ICOS^{+}$ Tfh and Tfr cells (sorted from NP-OVA in CFA immunized $Ctla4^{flox/flox}Foxp3^{ERT\text{-}Cre}$) and submitted to tamoxifen-induced depletion of Tfr cells an overall increase in Tfh cells and NP-specific antibodies (with lower affinity) was noticed[259]. In these experimental conditions, the emergence of low affinity antibodies might be due to a less stringent B cell competition imposed by a facilitated Tfh cell-dependent B cell help in the absence of CTLA-4-suficient Tfr cells. Another study reported that $Bcl\text{-}6^{flox/flox}Foxp3^{Cre}$ conditional knockout mice (specifically lacking Tfr cells) had normal GC size and GC B cell numbers, but exhibited high serum titres of antigen-specific IgA responses (and lower titres of antigen-specific IgG), suggesting that besides antigen specificity of antibodies produced within GC, Tfr cells modulate class-switch[303]. Finally, in the gut microenvironment, Kawamoto and colleagues demonstrated that Tfr cells are required in GCs of Peyer's patches to normalize the microbiota diversity, as transfer of Treg cells lacking Bcl-6 expression failed to induce normal bacterial diversity in T cell-deficient ($Cd3e^{-/-}$) mice[302]. Previous work of this group elucidated for the IgA dependency of gut microbiota and immune homeostasis[304–307]. Thus, they tested whether IgA production is impaired in the absence of Tfr cells. Indeed, IgA-producing B cells were decreased in small intestine *lamina propria*

(but not in Peyer's patches), directly linking gut Tfr cells with IgA production and microbiota homeostasis[302].

These regulatory mechanisms have been further studied in murine models of autoimmunity diseases, where Tfr cells were directly implicated in ensuring tolerance to self-antigens and preventing autoimmunity[273,278,282,283]. The loss of Tfr cells in $Stim1^{flox/flox}Stim2^{flox/flox}Cd4^{Cre}$ mice (described above) was followed by a massive increase in Tfh cells, accumulation of GC B cells, and fatal autoimmunity manifestations, such as elevated concentrations of serum autoantibodies (anti-dsDNA, anti-La, anti-Ro52, and anti-Ro60) and autoantibody-induced glomerulonephritis[283]. Tfr cell requirement for restraining antigen-specific GC responses *in vivo* was confirmed by Vaeth and colleagues. Using a LCMV infection model, they reported an amplified production of anti-LCMV antibodies when Tfr cells are absent (due to SOCE deficiency) in the recipient mice of transferred LCMV-specific (SMARTA) $CD4^+$ T cells[283]. In an independent set of experiments, the same group found that $Nfat2^{flox/flox}Cd4^{Cre}$ mice were more prone to develop the clinical hallmarks of SLE after induction of a SLE-like disease (by immunization with chromatin isolated from syngeneic-activated lymphocytes)[282]. Curiously, in murine models of chronic graft-versus-host disease (another disease known for the pathogenic importance of GC responses), CXCR5-exppressing Treg cells were required to treat this disease[308]. Botta and colleagues found that in the absence of Tfr cells (either by treating influenza infected mice with recombinant IL-2 treatment or by infecting $Bcl\text{-}6^{flox/flox}$Foxp3 conditional knockout mice) self-reactive anti-nuclear antibody producing plasma cells emerged from influenza-induced GC reactions[278]. These findings suggest that during GC reactions, IL-2 consumption acts as a rheostat that, while facilitating specific Tfh cell responses, selectively prevents Tfr cell development at the peak of the GC response. Thus, the selective use of Tfr cells (or IL-2 to fine-tune Tfr cell responses) to target diseases after the establishment of pathogenic GC reactions might be regarded as a new research direction.

The outcome of GC reactions is also modulated by age-induced alteration in Tfh and Tfr cell numbers and function[309]. In a study by Sage and colleagues, an increased proportion of Tfr cells (with increased Tfr/Tfh ratio) in aged (compared to young) mice following NP-OVA in CFA immunization was observed. Aged mice harboured reduced titres of NP-specific antibodies[309]. The authors used different approaches to address whether defective Tfh and/or Tfr cell functions contributed to this observation. They cocultured young and aged Tfr with

young Tfh and B cells sorted from NP-OVA immunized mice and further stimulated the cells *in vitro* with NP-OVA. Both young and aged Tfr cells suppressed class switch by B cells and Tfh cell proliferation[309]. To validate these findings, they adoptively transfer young and aged Tfr cells along with young Tfh cells to CD28-deficient mice (which cannot generate Tfh and Tfr cells). After 10 days of NP-OVA in CFA immunization, antigen-specific antibody titres were comparable between recipients of young and aged Tfr cells. While, aged Tfr kept their suppressive capacity, aged Tfh cells showed less antigen-specific stimulatory capacity[309]. Thus, defective antigen specific antibody responses in aging resulted, at least in part, from the combination of defective Tfh cell function and higher proportions of Tfr cell in aged mice.

GC reactions are orchestrated in secondary lymphoid organs in physiological conditions. The recent identification of circulating Tfr cells (see above) add an additional layer of complexity over the mechanisms of Tfr cell function, as compartments of the immune system might evoke different Tfr cell responses[196,197,223,258,260,310]. Sage and colleagues designed *in vitro* systems to address the function of Tfr cells derived from lymph nodes and blood. They first induced immune responses *in vivo* by immunizing mice with NP-OVA in CFA. Then, they sorted B, Tfh and Tfr cells from those immunized mice and stimulated them *in vitro* with anti-CD3 and anti-IgM[258,260]. Circulating Tfr cells had lower capacity to suppress B cell activation and class switch recombination in cocultures with Tfh and B cells[258]. Although, Sage and colleagues did not directly compare blood and lymph node Tfr cell function *in vivo*, they observed that blood Tfr cells were able to suppress B cell activation and production of antigen-specific antibodies (while Treg cells were not)[260]. For that they took advantage of adoptive cell transfers of blood Tfr cells (sorted from NP-OVA in CFA immunized mice) to $Cd28^{-/-}$ or $Tcra^{-/-}$ mice (which do not generate follicular T cells)[260]. Likewise, when blood Tfr cells from NP-OVA in CFA immunized CD45.2 mice were transferred to CD45.1 mice, blood Tfr (CD45.2-expressing cells) cells were readily seen in recipient lymph nodes and GC 7 days after NP-OVA immunization[258]. Hence, blood Tfr cells are phenotypically distinct from lymph node Tfr cells, but rapidly gain access to GC and suppress antigen-specific B cell responses upon immunization[258,260].

Control of Cytokine Milieu and Cell Metabolism

Sage and colleagues extensively studied Tfr cell function directly on Tfh and B cells using several *in vitro* systems (described above)[258–260,272]. In these systems, Tfh cells cultured in

presence of Tfr cells expressed lower levels of Ki-67, downregulated IFN-γ, IL-21, IL-10 and TNF-α, and produced less IL-4 and IL-21[258,272]. In another study, equivalent results were observed in Bcl-6-deficient Treg cells. In those mice, Tfh cells upregulated IFN-γ, IL-21, and IL-10 upon SRBC immunization[303].

At least part of the suppressive mechanism of Tfr cells seems to involve the modulation of metabolic pathways (specifically, oxidative phosphorylation, glycolysis and one-carbon metabolism), and metabolic checkpoints (such as MTORc1) in Tfh and GC B cells[272]. Sage and colleagues used the same *in vitro* systems described above to show that neither Tfh or B cells lost their transcriptomic signature or activation potential in the presence of Tfr cells, but did not express their effector molecules[272]. Indeed, B cells were still able to express early activation markers (such as CD69) and to proliferate in presence of Tfr cells. Additionally, the lack of specific effector molecules on Tfh and B cells was not due to cell exhaustion or anergy[272]. The effector molecules of B cells downregulated in presence of Tfr cells were Pou2af1 (required for GC B cell formation), Xbp1 (required for antibody secretion) and Aicda (required for class switch recombination)[137,272,311–313]. The modulation of B cells persisted in the absence of Tfr cells and was associated with epigenetic changes. However, the suppressive state induced by Tfr cells was reversible, as IL-21 addition was able to overcome the suppressive effect of Tfr cells by increasing B cell metabolism and by directly inhibiting Tfr cells (as described above) (**Figure 2**)[272]. While, the exact way Tfr cells induce these metabolic changes in Tfh and B cells is speculative, Sage and colleagues proposed that Tfr cells may physically interrupt bidirectional costimulation and linked recognition during Tfh cell – B cell immunological synapses (which are required for effector functions of both cells) (**Figure 3**)[272]. Alternatively, Tfr cells may use metabolic disruption mechanisms used by Treg cells, such as generation of pericellular adenosine through the action of ectoenzymes CD39 and CD73 or transfer of the potent inhibitory second messenger cAMP into effector T cells through membrane gap junctions[209–213]. The suppression actions of adenosine are of particular interest in the context of humoral responses, as adenosine can inhibit IL-6, a cytokine required for Tfh cell differentiation[61,78,212]. Curiously, a recent report found that adenosine A2a receptor agonists can limit GC and Tfh cell responses following immunization, suggesting adenosine may indeed be a mediator of Tfr cell effector functions in humoral responses[314].

Molecular Mechanisms of Tfr Cell Function

CTLA-4 has a widely known function in maintaining immune homeostasis and mediating Treg cell function[155,214–216]. Indeed, mice selectively lacking CTLA-4 expression on Treg cells succumbed to a spontaneous lymphoproliferation with fatal T cell-dependent autoimmune disease[214,217]. Briefly, CTLA-4 works in a cell-extrinsic manner by capturing ligands (namely CD80 and CD86) from the surface of APCs, thereby limiting CD28 engagement. In a highly endocytic behaviour, CTLA-4 can strip APCs of costimulatory ligands, traffic them to lysosomes for degradation, and then recycle to repeat[215,216,315].

As CTLA-4 expression on Tfr cells was comparable to its expression on activated highly suppressive Treg cells (as described above), two independent groups extensively studied the role of CTLA-4 in Tfr cell function[259,269]. Tamoxifen-induced CTLA-4 depletion on Treg cells at the time of immune challenge was used by Sage and colleagues to demonstrate that GC B cells, Tfr and Tfh cells expanded simultaneously (but with relative greater increase in Tfr cells) following immunization with NP-OVA in CFA[259]. While, transendocytosis and downregulation of CD80 and CD86 has been proposed to be one mechanism by which CTLA-4 controls immune responses, these authors did not observe any difference on CD80/CD86 staining in B cells upon CTLA-4 depletion[259,315]. Importantly, CTLA-4-deficient Tfr cells were less capable to suppress class-switch recombination to IgG1, and Tfh cell proliferation[259]. Concordantly, Wing and colleagues showed that in the absence of CTLA-4 in Treg cells a massively enhanced GC and Tfh cell responses occurs. In these CTLA-$4^{flox/flox}$Foxp3^{Cre} hemizygous male mice, Treg and Tfr cells were also increased suggesting that in the absence of CTLA-4 even high proportions of regulatory T cells are unable to restrain humoral responses[269]. To circumvent a large scale inflammatory milieu, the authors also studied CTLA-$4^{wt/flox}$Foxp3^{Cre} hemizygous male mice. In this context, antigen-specific Tfh and B cells (but not NP-specific IgG titres) were increased following NP-OVA in alum immunization. To address the mechanisms of CTLA-4 on humoral responses, the authors took advantage of female mice homozygous for CTLA-4^{flox} and heterozygous for Foxp3^{Cre} to sort CTLA-4-sufficient and CTLA-4-deficient Treg and Tfr cells from otherwise healthy mice. Contrary to the *in vivo* experiments reported by Sage and colleagues, CTLA-4-deficient Treg and Tfr cells failed to downregulate CD86 and CD80 on activated B cells *in vitro*[269]. Additionally, they found that upon CTLA-4 blockage IL-4 producing Tfh cells increased[269]. Thus, the authors proposed that CTLA-4 expressed on Tfr cells restrains IL-4 secretion by Tfh and blocks CD80/CD86 costimulatory signals on B

cells, limiting antigen-specific humoral responses[269]. Thus, CTLA-4 has been regarded as one key mediator of Tfr cell regulation of humoral responses (**Figure 3**).

Despite most of Tfr cell suppressive capacity is lost in the absence of CTLA-4, it is expected that these cells employ multiple and complementary regulatory mechanisms (such as TGF-β, IL-10 and granzyme B secretion), like other Treg subsets (**Figure 3**) [28,152,153,155,196,257,297]. The role of IL-10 and TGF-β in Tfr cell-mediated suppression is largely unknow. The few attempts to study the role of IL-10 in Tfr cell function produced mixed results. While, Tfr cells produce IL-10 gene transcripts, IL-10 production assessed in supernatants of cocultures with Tfh and B cells was reduced when Tfr cells were added, suggesting that Tfr cells might not be able to rise the IL-10 concentration near Tfh cell – B cell synapses[258,255]. Moreover, it is unlikely that IL-10 mediates Tfr cell suppression within GC, as IL-10-deficient mice have relative normal levels of antibodies and mice with IL-10-deficient Treg cells did not developed systemic autoimmunity[200,316,317]. Tfr cells also express granzyme B (although in lower levels than Treg cells), thus granzyme B-mediated cytolysis might be another regulatory mechanism employed by Tfr cells, as it was already described for Treg cells (see above)[164,255].

Division of Labour Between Treg and Tfr Cells

Although the main biological aspects of Treg cells have been broadly established, it is still unclear how different Treg cell subsets integrate to underpin immune tolerance and regulation of humoral responses. Many studies demonstrated that in the absence of $Foxp3^+$ Treg cells uncontrolled and spontaneous humoral responses arise[318–321]. Whether those findings resulted from the absence of all Treg cell subsets or uniquely from the absence of $CXCR5^+$ Tfr cells is not known. Nevertheless, humoral suppressive capacity has been claimed to be assigned uniquely to Tfr cells. This concept arose from Sage and colleagues observations that conventional (non-Tfr) Treg cells lack the capacity to suppress Tfh cell proliferation, B cell activation, and class switch recombination[258]. Likewise, conventional Treg cells did not fully abrogate the secretion of IgG (contrary to Tfr cells)[272]. To validate these *in vitro* findings, Sage and colleagues transferred Tfh and Tfr (or Treg) cells (sorted from NP-OVA in CFA immunized mice) to $Tcra^{-/-}$ mice. In these lymphopenic conditions and using 1:2 non-physiological Treg/Tfh ratio, they claimed conventional Treg cells were not able to block NP-specific IgG secretion by B cells[260]. Conversely, two independent groups found a comparable decrease in Tfh cell proliferation when co-cultured with Tfr and

conventional Treg cells[254,269]. Hence, while a direct comparison of Tfr and conventional Treg in physiological conditions is lacking, Tfr cells presumably acquire their unique humoral suppressive capacity when they co-opt Tfh cell differentiation program. The suppression of Tfh cell proliferation is probably not unique to Tfr cells, as it might be a general Treg cell feature. It is noteworthy that migration toward CXCL13 enriched B cell zones is only possible for CXCR5-expressing cells. Therefore, one critical aspect that might distinguish Tfr from conventional Treg cells *in vivo* is the exceptional ability of Tfr cells to get access to GCs.

3.3.3. Tfr Cells in Humans

As described above, Tfr cell biology has been mainly studied in murine models. However, there is a growing concern that laboratory mice do not reflect many relevant aspects of the human immune system[322]. Therefore, studies directly performed in humans are vital to understand Tfr cell biology in the complex human immune system.

So far, it has been shown that, Tfr cells in human lymph nodes do not require B cells for their maintenance, as depletion of $CD20^+$ B cells by rituximab did not decrease the number of $CD57^+CXCR5^+Foxp3^+CD127^-$ Tfr cells[323]. Similarly to murine findings, there is evidence suggesting some divergence in human Tfr and Tfh cells differentiation: a primary immunodeficiency defined by PI3KR1 gain-of-function mutation did not affect the frequency of tonsil Tfr cells, while it significantly reduced the frequency of Tfh cells[324].

The restricted access to human tissues forced the search for putative Tfr cells in human blood. Several studies confirmed circulating $CXCR5^+$ T cells as the counterparts of tissue Tfh cells, arising from lymphoid tissue cells before reaching the GC, in spite of low Bcl6 expression[53–56,120]. It is well known that some human blood Treg cells express CXCR5 among other CXC chemokine receptors[325]. Thus, many studies have been using circulating $CXCR5^+Foxp3^+$ T cells to define Tfr cells in humans (see below). Surprisingly, no study specifically addressed whether blood $CXCR5^+Foxp3^+$ T cells are truly circulating GC Tfr cell counterparts. Although circulating memory Tfr cells were observed after immunization protocols in mice[258,260], CXCR5 and Foxp3 transient upregulation upon human T cell activation challenge the assumption that human blood $CXCR5^+Foxp3^+$ T cells are Tfr cells[49,235,326–328]. Dhaeza and colleagues found that blood $CXCR5^+PD\text{-}1^+CD25^+CD127^-$ T

cells increased on day 7 following influenza vaccination (showing a positive correlation with plasma antibodies titers for influenza), and suppressed T cells *in vitro* upon αCD3/CD28 stimulation[329]. Although this report has correlated blood $CXCR5^{+}PD\text{-}1^{+}CD25^{+}CD127^{-}$ T cells with influenza vaccination responses, they did not provide any evidence of an intrinsic humoral regulatory capacity. The results described in the first part of the results of this thesis addressed this specific issue providing the first evidence of the biology and ontogeny of human blood $CXCR5^{+}Foxp3^{+}$ T cells from a Tfr cell perspective[330].

Based on these limited studies some aspects of Tfr cell biology are now being clarified (**Table 4**).

Table 4: Biology of Tfr cells in Mice and Men
Adapted from Maceiras *at al*[331]

	Mice	**Men**
Membrane markers	CD25, CXCR5, PD-1, ICOS, CTLA-4	CD25, CXCR5, PD-1, ICOS (?)
Transcription factors	Foxp3, Bcl-6, Blimp-1, NFAT2	Foxp3, Bcl-6
Immune compartments	Lymph node, spleen, blood	Tonsil, lymph node, spleen, blood, ectopic lymphoid structures
Cell of origin	Thymic Treg cells	*Not directly studied*
Differentiation signals	MHCII, CD28, ICOS, PD-1, SAP, IL-21, SOCE, TRAF3, STAT3	IL-21. *Other molecules not directly studied.*
Functions	– Control Tfh and GC B-cell numbers by inducing a suppressive state and inhibiting proliferation – Impair IL-4 and IL-21 production by Tfh cells – Reduce the levels of antibodies produced (namely IgM and IgG) and class switch recombination – Control antibodies' quality	– Impair Tconv and Tfh cell proliferation – Impair IL-4 and IL-21 production by Tfh cells – Impair immunoglobulin (namely IgA) production by B cells
TCR Repertoire	Tfr cells have an oligoclonal TCR repertoire, different from Tfh cell repertoire, and similar to the repertoire of Treg cells.	*Unknown*

3.3.4. Tfr Cells in Different Human Diseases

Despite the speculative role of human blood $CXCR5^+Foxp3^+$ Tfr cells as a specialized subset of Treg cells in regulating humoral responses, recently many groups reported altered Tfr cell composition in peripheral blood of patients with several diseases (**Table 5**).

Table 5: $Foxp3^+$ Tfr cells in Human Diseases
Adapted from Maceiras *at al*[331]

Reference	Clinical Condition	Tissues	Tfr cells	Major findings
Faghih *et al*, 2014[332]	Breast cancer	Lymph nodes	$CXCR5^+$ $Foxp3^+$ $Bcl6^+$ $CD4^+$	No impact on Tfr cell frequency.
Wallin *et al*, 2014[323]	Kidney transplant recipients undergoing therapy with Rituximab	Lymph nodes	$CD57^+$ $CXCR5^+$ $Foxp3^+$ $CD127^-$ $CD4^+$	Rituximab had no impact on Tfr cells. Tfr cells reduced IgA production by B cells (*in vitro*).
Wang *et al*, 2014[333]	Chronic hepatitis B (HBV) and C (HCV)	Blood	$CXCR5^+$ $Foxp3^+$ $CD4^+$	Tfr cells increased during HBV and HCV infection. Tfr cells positively correlated with HBsAg titres and viral load.
Chen *et al*, 2015[334]	*S. japonica* infection	Blood	$CXCR5^+$ $Foxp3^+$ $CD4^+$	Tfr cell frequency increased during *S. japonica* infection.
Colineau *et al*, 2015[335]	HIV-1 infection	Spleen	$Foxp3^+$ $CXCR5^+$ $CD45RA^-$ $CCR7^-$ $CD4^+$	Tfr cell frequency increased during HIV infection.
De Bruyne *et al*, 2015[336]	Food alergy	Blood	$CXCR5^+$ $CD45RO^+$ $Foxp3^+$ $CD25^+$ $CD4^+$	No impact on Tfr cell frequency.
Dhaeze *et al*, 2015[329]	Multiple sclerosis (MS)	Blood	$CXCR5^+$ $PD\text{-}1^+$ $CD25^+$ $CD127^-$ $CD4^+$	Tfr cell frequency increased upon influenza vaccination, positively correlating with specific antibody titres. Tfr cell frequency was decreased in MS, showing less suppressive capacity.
Miles *et al*, 2015[337]	HIV-1 infection *In vitro*	Lymph nodes Tonsil cells	$CD25^+$ $CD127^-$ $CXCR5^+$ $CD3^+$ $CD8^-$	Tfr cell expanded upon HIV infection (TGF-β dependent). Tfr cells reduced $ICOS^+IL\text{-}21^+IL\text{-}4^+$ Tfh cells.

Shan *et al*, 2015[338]	Ankylosing spondylitis (AS)	Blood	CXCR5$^+$ Foxp3$^+$ CD4$^+$	Tfr cells were increased in AS, increasing further upon therapy. Tfr cells negatively correlated with serum IgA titres.
Di Fonte *et al*, 2016[324]	PI3KR1 gain-of-function (c.1425 + 1G>T)	Tonsil	CXCR5$^+$ Foxp3$^+$ CD4$^+$	No impact on Tfr cell frequency.
Moody *et al*, 2016[339]	HIV-1 infection	Blood	CXCR5$^+$ CD25$^+$ Foxp3$^+$ CD4$^+$	Tfr cells were increased in HIV infected patients
Xu *et al*, 2016[340]	Kidney allograft rejection	Kidney	CXCR5$^+$ Foxp3$^+$	Tfr cells were rarely present in kidney tertiary lymphoid structures.
Vaeth *et al*, 2016[283]	ORAI1 p.R91W	Blood	CD45RO$^+$ Helios$^+$ Foxp3$^+$ CD4$^+$	SOCE signalling defects impaired Tfr cell frequency.
Wen *et al*, 2016[341]	Myasthenia gravis (MG)	Blood	CXCR5$^+$ Foxp3$^+$ CD4$^+$	Tfr cells were decreased in MG, inversely correlating with disease severity. Treatment with steroids re-establish the frequency of Tfr cells.
Chen *et al*, 2017[342]	Kidney allograft rejection	Blood Kidney	CXCR5$^+$ ICOS$^+$ Foxp3$^+$ CD127$^-$ CD4$^+$	Tfr cells were decreased in patients with antibody-mediated rejection. Tfr cells inhibited B cell proliferation and IgA and IgG production. Sirolimus induced a decrease in Tfr cell frequency.
Cobb *et al*, 2017[343]	HCV	Liver	CXCR5$^+$ PD-1$^+$ Foxp3$^+$ CD25$^+$ CD4$^+$	Liver Tfr cells were increased in HCV infected patients. Tfr cell expansion was induced by TGF-β-containing exosomes derived from HCV-infected hepatocytes. Tfr cells restrained Tfh cell proliferation.
Cunill *et al*, 2017[344]	Common Variable Immunodeficiency (CVID)	Blood	CXCR5$^+$ CD25$^+$ CD127$^-$ CD4$^+$	Tfr cells were reduced in CVID patients with < 2% of IgD$^-$CD27$^+$ B cells. Tfr cells suppressed conventional T cell proliferation.
Fonseca *et al*, 2017[330]	Sjögren's syndrome (SS) Healthy	Blood	CXCR5$^+$ Foxp3$^+$ CD25$^+$ CD4$^+$	Tfr cells were increased in SS patients with serum autoantibodies. Blood Tfr cells are immature cells, not fully licensed with humoral suppressive function (*in vitro*). Blood Tfr cells emerge prior to B cell interactions.
Miller *et al*, 2017[345]	HIV-1 infection *In vitro*	Tonsil cells	CXCR5$^+$ CD25$^+$ CD127$^-$ CD3$^+$ CD8$^-$	Tfr cells expressed CCR5 and Ki-67 at baseline. Tfr cells were the most permissive cell type for R5-tropic HIV1 infection.

Autoimmune diseases

So far, very few studies addressed Tfr cells in human autoimmunity. Blood CXCR5$^+$PD-1$^+$CD25$^+$CD127$^-$ Tfr cells were found reduced in MS patients[329]. In addition, blood Tfr cells

from MS patients were less suppressive than equivalent cells sorted from healthy controls, using $CD25^{-}CD127^{+}$ T cells as responders *in vitro*[329]. This work suggests that blood Tfr cells might be functionally defective in autoimmunity. However, the functional assays performed in this study did not address the putative specialization of Tfr cells in suppressing humoral responses. An additional study also found a decreased frequency of blood $CXCR5^{+}Foxp3^{+}$ Tfr cells in untreated patients with MG, recovering to normal levels after corticosteroid-based treatment[341].

However, not all studies of $CXCR5^{+}Foxp3^{+}$ T cells in autoimmunity are concordant. A higher frequency of blood $CXCR5^{+}Foxp3^{+}$ Tfr cells was found in patients with AS, increasing even further after treatment with the TNF blocker, Etanercept[338]. The blood frequency of Tfr cells did not correlate with MS nor AS disease severity, questioning the direct role of blood $CXCR5^{+}Foxp3^{+}$ T cells in autoimmune pathogenesis. In SLE, Xu and colleagues described a reduced frequency of blood $CXCR5^{+}CD25^{+}CD127^{-}$ Tfr cells, although, they did not use Foxp3 for identifying those cells[346]. Blood Tfr cell population, which was enriched for $PD\text{-}1^{+}ICOS^{+}$ cells, negatively correlated with anti-dsDNA antibodies, disease activity score, and serum IL-21 concentration, but not with total IgG or frequency of blood plasmablasts. Interestingly, patients with relapsing SLE showed an increase in Tfr cell frequency after treatment[346]. While, this work suggests that Tfr cells are decreased in blood of patients with chronic systemic autoimmune diseases, it is noteworthy that treatment regimens at baseline were not considered during data analysis. Whether reduced Tfr cells in blood was due to SLE or to immunosuppressant drugs is not known.

Overall, those studies showed that blood Tfr cells are reduced in human autoimmune diseases. As their relationship with disease activity, autoantibody production, and treatment regimens was not consistent in the different studies, the biological role of Tfr cells in autoimmunity remains elusive.

HIV and Other Infectious Diseases

HIV-1 infected patients (not receiving highly active antiretroviral therapy) have an increased frequency of GC $Foxp3^{+}CD4^{+}$ Tfr cells[337], spleen $Foxp3^{+}CXCR5^{+}CD45RA^{-}CCR7^{-}CD4^{+}$ Tfr cells[335], and blood $CXCR5^{+}CD25^{+}Foxp3^{+}CD4^{+}$ Tfr cells[339]. Tfh cells (defined as $CXCR5^{+}CD45RA^{-}CCR7^{-}CD4^{+}$ T cells for spleen and as $PD\text{-}1^{+}CXCR3^{-}CXCR5^{+}$ $CD4^{+}$ T

cells for peripheral blood) were also increased, suggesting that Tfr and Tfh cell expansion can occur simultaneously in cases of antigen persistence.

In vitro studies using tonsil cells spinoculated with X4 and R5 HIV-1 have shown Tfr cell expansion (with increased CTLA-4, LAG3 and IL-10 expression) upon HIV infection in a TGFβ-dependent manner[337]. It remains to be determined whether induced Treg cells are physiologically present *in vivo* as functionally competent suppressive cells in humans, Miles and colleagues confirmed (in line with murine studies described above) that $CXCR5^{+}CD25^{-}$ Tfh cells did not acquire Foxp3 expression upon *in vitro* stimulation, even in the presence of TGFβ[171,328,337,347,348]. These authors also demonstrated that in HIV-1 infected tonsil cells, a predominance of indoleamine-2,3-dioxygenase producing plasmacytoid DCs, known for their role in Treg cell generation, were increased, indicating specific DCs subsets may be more proficient in promoting Tfr cell fate[27,349–351]. In the context of *in vitro* HIV-1 infection, Tfr cells impaired ICOS expression and proliferation of Tfh cells, as well as their IL-21 and IL-4 secretion capacity[337]. Tfr cells were also found to be the most permissive $CD4^{+}$ T cell subset to R5-tropic HIV-1 infection both *in vitro* and *in vivo*[345]. Again, with tonsil cells spinoculated with X4 and R5 HIV-1, Miller and colleagues demonstrated that $CXCR5^{+}CD25^{+}CD127^{-}CD3^{+}CD8^{-}$ Tfr were the most permissive cells for R5-tropic HIV-1 internalization. This finding was not due to acquisition of Tfr cell markers upon HIV-1 infection, but owed to expansion of pre-existent Tfr cells[337,345]. Additionally, they showed that Tfr cells from lymph nodes of HIV-1 infected patients had the highest number of HIV-1 copy number per ng of total RNA[345]. They claimed high proliferative rate and high CCR5 expression of Tfr cells could drive this permissivity[345]. In blood, the presence of broad neutralizing antibodies did not impact the frequency of Tfr cells, although patients with high titres of neutralizing antibodies displayed a higher expression of PD-1 in Tfr cells[339]. Although increased PD-1 signalling has been shown to inhibit Tfr cell function in mice[260], it is still highly speculative to correlate the presence of broad neutralizing antibodies with putative Tfr cell exhaustion.

Blood $CXCR5^{+}Foxp3^{+}$ Tfr cells were also found increased in HBV and HCV chronically infected patients, showing a significant correlation with blood viral load in both infections. Another group studied Tfr cells in cell suspensions obtained from HCV infected livers[343]. $CXCR5^{+}PD\text{-}1^{+}CD25^{+}Foxp3^{+}$ Tfr cells, as well as Tfh cells, were increased in the liver of HCV infected patients. Then, Cobb and colleagues used *in vitro* co-cultures with human

hepatoma cell lines to demonstrate that HCV-infected hepatocyte induced Tfr cell expansion (with higher IL-10 and CTLA-4 expression)[343]. They claimed HCV-infected hepatocytes were able to expand highly suppressive Tfr cells, yet they did not compare Tfr cells derived from HCV-infected hepatocytes co-cultures with Tfr cells derived from non-infected control cultures[343]. Curiously, when Cobb and colleagues blocked TGF-β on exosomes derived from HCV-infected hepatocytes the Tfr cell expansion effect was lost[343]. In recent years, the modulation of immune system by exosomes (and extracellular vesicles), and its therapeutic potential, became one area of active research[202]. Thus, the work of Cobb and colleagues (although performed in very artificial systems) disclosed one putative mechanism by which exosomes may regulate humoral immune responses in the context of HCV infection. An increased frequency of blood $CXCR5^{+}Foxp3^{+}CD45RA^{-}$ Tfr cells was also found in helminthic infection by *Schistosoma japonica*[334].

Primary Immunodeficiencies

Many human monogenic primary immunodeficiencies are associated with defective B cell responses. It is the case of STAT3, IL-10R, CD40L, NEMO, BTK, and ICOS mutations where, as anticipated, there is a decreased frequency of Tfh cells[119]. Although, frequency of blood $CXCR5^{+}Foxp3^{+}$ Tfr cells were not yet studied in these pathologic conditions, patients with less than 2% of $IgD^{-}CD27^{+}$ B cells in the setting of Common Variable Immunodeficiency (CVID) have a reduction of blood $CXCR5^{+}CD25^{hi}CD127^{low}$ Tfr cell frequency, in line with a reduction of total Treg frequency in peripheral blood[344]. While, this study suggests a relationship between $IgD^{-}CD27^{+}$ B cells and blood Tfr cells, the clinical heterogeneity and largely unknown molecular mechanisms driving CVID preclude a definite conclusion about blood Tfr cell ontogeny[352]. Specifically, gene defects known to cause CVID (such as, genes encoding CTLA-4, ICOS, IL-21, and IL-21R) can directly affect Tfr cell differentiation and function (**Figure 1 and 2**)[259,269,270,352].

Recently, the SOCE (store-operated calcium entry) pathway in T cells has been implicated in Tfr cell differentiation in humans. Patients with severe combined immunodeficiency-like disease due to inherited loss-of-function mutations in ORAI1 and STIM1 (genes that abolish SOCE) have a significant reduction in blood $CD45RO^{+}Helios^{+}Foxp3^{+}$ Tfr-like cells[283]. In another recent study, IL-21R-deficiency patients have been shown to have a significant increase in frequency of blood $Foxp3^{+}CXCR5^{+}PD\text{-}1^{+}$ Tfr cells. In contrast, a marked decrease in circulating $CXCR5^{+}PD\text{-}1^{+}$ Tfh cells was observed in IL-21R-deficiency

patients[270]. Taken together, these recent studies suggest that human Tfh and Tfr cells have different, sometimes reciprocal, requirements for their differentiation.

Transplantation

In renal transplant patients with acute and chronic rejection, very rare $CXCR5^{+}Foxp3^{+}$ Tfr cells were found within graft tissues. Xu and colleagues claimed Tfr cells (as well as Treg cells) were not responsible for tolerance in ectopic lymphoid structures (ELS) found in the graft tissues, as the presence of Tfr cells was not associated with longer duration of graft function[340]. More recently, blood $CXCR5^{+}ICOS^{+}Foxp3^{+}CD127^{-}$ Tfr cells and tissue (renal allograft sections) $CXCR5^{+}Foxp3^{+}$ Tfr cells were associated with antibody-mediated rejection in renal transplant patients[342]. Using unspecified *in vitro* conditions, a decreased frequency of recovered Tfr cells was found when peripheral blood lymphocytes (sorted from healthy donors) were cultured under the presence of Sirolimus (compared to Cyclosporine and Tacrolimus)[342]. This finding is not consistent with NFAT2-dependency of Tfr cell differentiation, as Sirolimus blocks mTOR and not NFAT (like Cyclosporine and Tacrolimus do)[282,283,353]. Chen and colleagues speculated that Tfr cells directly inhibited B cell proliferation and IgG and IgA production in a CTLA-4, IL-10 and TGF-β-dependent manner[342]. Curiously, the author did not define the stimulation conditions for B cell proliferation and activation. In contrast, the same researches, in another set of experiments concluded Tfr cells from patients with antibody-mediated rejection had no functional defect[342].

4. T – B Cross-Talk in Autoimmunity

The concept of autoimmunity was first predicted by Nobel laureate Paul Ehrlich at the start of the twentieth century with the definition of "horror autotoxicus". His experiments led him to conclude that the immune system is normally focused on responding to foreign agents and has an inbuilt tendency to avoid attacking self-tissues. However, the occasional failure to distinguish friends from foes results in a coordinated attack of self-tissues resulting in autoimmune diseases. The perplexing issue of what allows the immune system to attack self-tissues is still an active focus of research. At a biochemical level, no structural difference exists between self and foreign antigens. Autoreactive immune cells can, and do, arise

against any tissue, yet the prevalence of autoimmunity remains low because additional molecular, cellular and contextual cues control immune responses towards self-antigens.

More than 80 distinct autoimmune diseases have been described and many of these are associated with the development of autoantibodies. Indeed, detection of autoantibodies in patients' serum is used in the clinical diagnosis of many autoimmune diseases. Some studies suggested that autoantibodies share a common ontogeny with conventional antibodies that are formed during T cell-dependent responses to foreign antigens or indeed as a consequence of somatic hypermutation in GCs[15,354,355]. Several strains of mice that typically develop autoimmune diseases exhibit spontaneous GC formation providing further evidence that GC reactions are involved in the pathogenesis of autoimmune diseases[143]. Moreover, in organ-specific autoimmune diseases, in which expression of the target self-antigen is often tissue restricted, organized ELS (also called tertiary lymphoid organs) harbouring abnormal self-centred GC reactions, form at the site of tissue pathology (see below)[143,356,357].

Thus, B – T crosstalk is a ubiquitous phenomenon in immune responses driving autoantibody-mediated autoimmune diseases. In the last years, targeting various critical molecules involved in pathological pathways of autoimmune diseases has led to disease modulation. However, most of these new immune therapies target cytokines or individual cell subsets in non-rate-limiting processes. While, few therapies, such as anti-TNF monoclonal antibodies, have markedly ameliorated some autoimmune disease progression, most of the available drugs have little benefit and/or serious adverse effects in autoimmune diseases[358]. Targeting continuous self-centred B – T crosstalk might selectively disrupt autoantibody formation and prevent B-cell lymphomagenesis[143,356,357,359].

4.1. Dysregulation of Germinal Centre Responses

Pathological autoantibodies are mainly dependent on GC reactions, as the majority are high affinity IgG antibodies[143]. The stochastic nature of somatic hypermutation makes the generation of autoreactive B cell clones an almost certain by-product of routine GC responses to foreign antigens[15,360,361]. If these autoreactive B cell clones are not effectively eliminated or at least regulated, there can be long-term consequences in the form of autoimmune diseases[143]. Therefore, dysregulated GC responses are an important component in the pathogenesis of autoimmunity disorders.

A wide range of murine studies have identified several mechanisms responsible for breaking GC tolerance in autoimmunity[143]. On the B cell side, TLR7 and TLR9 signalling, induced by the large amounts of self-DNA and RNA exposed by apoptotic GC B cells can overcome the need for cognate Tfh cell help, thereby driving the selection of autoantibody-producing B cell clones[362–364]. Failure of pro-apoptotic instructions (defined by downregulation of Bcl-2 and Bcl-X_L, and upregulation of BIM) in low affinity and autoreactive B cells may also promote their maintenance and development[365–368]. Finally, altered regulation of BCR signalling in GC B cells by low-affinity Fc receptors (FcγRIIB), which work by coupling the BCR to an inhibitory pathway, was also found to account for autoantibody-mediated disorders[369–371]. Insufficient disposal of apoptotic bodies by DNaseI, receptor tyrosine kinase MER, transglutaminase 2, complement component C1q and peroxisome proliferator-activated receptor-δ compromise the clearance of apoptotic cells leading to autoantibody formation[372–376]. On the Tfh cell side, overexpression of OX40L, TLR7 or Tfh cell intrinsic Roquin mutations contribute to aberrant positive selection of autoreactive B cell clones in murine models of autoimmune diseases[377–380]. The abnormal positive selection of autoreactive GC B cells may also be perpetuated by deficient CD95/CD95L signalling[381–383]. Additionally, excessive production of IL-21 may further contribute to GC longevity and emergence of autoantibody responses in those animal models[273,274,379].

Although, the role of somatic hypermutation and antigen-driven affinity maturation is well established in mouse and human autoimmunity, the actual contribution of defective GC-based checkpoints to the generation of autoantibody-mediated disorders is very difficult to establish in humans[143]. Nevertheless, multiple lines of evidence, namely from studies with SLE patients, support the role for abnormal GC reactions in the pathogenesis of human autoimmune diseases. First, the use of CD40L-specific antibodies ameliorates SLE disease severity and autoantibody titres, suggesting a follicular origin of autoantibodies[384,385]. Moreover, defective free apoptotic bodies clearance in SLE patients was associated with the induction of pathogenic autoantibodies[386,387]. Notably, the study of autoreactive 9G4 B cells have demonstrated that GCs in healthy individuals are competent in censoring these cells. By contrast, a high proportion of tonsil GCs from patients with SLE showed expansion of 9G4 B cell clones[388,389]. These findings suggest that defective GC-based tolerance checkpoints take part in the pathogenesis of human autoimmune diseases[143]. Lastly, an increasing number of studies have shown aberrant Tfh cell responses in patients with different autoimmune diseases[143,390–392].

Autoimmunity of follicular origin poses a considerable challenge because once the long-lived autoreactive B cells have been generated, treatments that disrupt GCs might fail. As the treatment of human autoimmune diseases often occurs years after the onset of the pathogenic process, combined therapeutic approaches targeting ongoing autoreactive GC reactions and the autoreactive long-lived B cell compartment may be an ideal option for those patients. However, to accomplish this aim, a GC "tune down" approach would be necessary in order to reduce the risk of autoimmunity while preserving foreign antigen-specific responses. Following the discovery of Tfr cells as GC "fine tune" regulators (as described above), targeting Tfr cell responses may constitute a novel and highly selective approach to the treatment of autoantibody-mediated autoimmune diseases. Yet, the still embryonic knowledge about Tfr cell biology in humans halts these advances.

4.2. Ectopic Lymphoid Structures

Tissues harbouring the target antigens of chronic immune responses are usually infiltrated by cellular effectors of the immune system, mainly T cells and macrophages, but also APCs, B cells, and plasma cells. These cellular elements often organize themselves anatomically and functionally as in secondary lymphoid organs, leading to *de novo* and ectopic formation of B cell follicles and T cell areas (ELS). This phenomenon is termed lymphoid neogenesis or tertiary lymphoid organ formation[356,357,393]. The architecture organization of ELS resemble the follicular compartment seen in secondary lymphoid organs, ranging from simple aggregates of B and T cells to highly ordered structures. The most complex ELS develop high endothelial venules (specialized postcapillary venous swelling that enable access to lymphocytes expressing L-selectin) and networks of stromal-derived FDCs[356,357]. Importantly, AID expression with evidence of *in situ* B cell affinity maturation and clonal selection characterizes these highly organized ELS[357]. However, contrary to secondary lymphoid organs, ELS lack afferent lymphatics, thus their formation is presumably driven by antigen diffusing from the surrounding inflammatory milieu[143].

The formation of ELS is not exclusive to autoimmune diseases, as it has also been described in chronic infections, graft rejection and cancer[356]. Following the demonstration of *in situ* pathogenic autoantibody production in ELS by Armengol and colleagues, compelling evidence supports the importance of ELS in human autoimmune diseases, namely in Rheumatoid Arthritis (RA), SS, SLE, MS, Primary Sclerosing Cholangitis, Primary Biliary

Cirrhosis, Hashimoto's Thyroiditis, and MG[356,357,394].

4.2.1. Development of Ectopic Lymphoid Structures

The mechanisms that regulate formation, maintenance and function of ELS are largely shared with those involved in the organogenesis, maintenance and function of secondary lymphoid organs[395,396]. In inflamed tissues, stromal/mesenchymal cells may acquire lymphoid tissue organizer cell-like properties, such as secretion of IL-7, RANKL and CXCL13. Indeed, some studies found that even in the absence of secondary lymphoid organs, the expression of chemokines in non-lymphoid organs is essential for local B and T cell responses and for the development and organization of ELS[397–399]. Those activating signals induce the migration of lymphoid tissue inducer and initiator cells. The interaction between membrane-bound heterotrimeric LTβ (LTα1β2) expressed by these cells with LTβ receptor expressed by mesenchymal cells triggers the subsequent development of ELS via CXCL13, CCL19 and CCL21 secretion[356,357,396]. At this stage, the formation of the lymphotoxin-lymphoid chemokine feedback loop is also required for the development of high endothelial venules[400]. Following this homeostatic chemokine release, an increasing number of $CCR7^+$ (which engage CCL19 and CCL21) T cells, as well as other T and B cell subsets, will progressively infiltrate the ELS[356,357,395,401]. In the specific inflammatory milieu driving ELS in autoimmune diseases, IL-17/IL-23 pathway was also found to contribute for ELS formation and maintenance[356,357]. Both IL-17 producing adult innate lymphoid cells (ILC3 cells) and Th17 cells were found to contribute to ELS development[402,403]. In addition to IL-17, IL-22 was reported to be required for the development of ELS that formed in salivary glands in a mouse model of virus-induced lymphoid neogenesis (which displays several features of SS). In that experimental model, IL-22 promoted CXCL13 secretion by exocrine gland stroma cells, thereby inducing ELS development[404]. Consistently, another report found that IL-22-deficient mice were protected from ELS development and autoimmunity through reduced B-cell recruitment to salivary glands[405].

The presence of ELS is often a transient feature of inflamed tissues, thus, the mechanisms that control their maintenance during disease may have greater clinical significance. Some reports have implicated Tfh cell responses and IL-21 in ELS development in patients with RA and SS[406,407]. While, known mechanisms of Tfh cell function in humoral responses formed in lymphoid organs may prompt the assumption that Tfh cells are key mediators of

maintenance and acquisition of GC features by ELS, this is still too speculative. However, other T helper cell subsets can contribute to GC reactions within ELS. Indeed, a IL-21-producing $CXCR5^{-}PD\text{-}1^{+}$ T cell population (called peripheral T helper cells) was found within ELS formed in synovial tissues of RA patients and proposed to be uniquely poised to promote B-cell responses and antibody production within inflamed non-lymphoid tissues[408]. Recently, IL-27-deficient mice were found to develop a more severe form of antigen-induced arthritis, characterized by the development of multiple ELS. In parallel, levels of IL-27 in synovial tissue from patients with AR correlated inversely with ELS development[409]. These findings suggest that IL-27 may be a negative regulator of ELS formation and/or maintenance[357]. Whether other negative regulators, such as Tfr cells, also play a role in ELS maintenance and function is not known.

4.2.2. Autoantibody Formation in Ectopic Lymphoid Structures

Some studies have shown, conclusively, that ELS in inflamed tissues retain the necessary molecular machinery to support *in situ* antibody diversification within ectopic GC reactions[356,357]. Microdissection of ectopic GCs isolated from synovium of patients with RA and salivary glands of patients with SS has demonstrated that ELS were populated by B cell clones with highly somatically hypermutated antibodies[410–412]. Indeed, intra-tissue AID-dependent clonal diversification and differentiation into antibody-producing cells can occur within ectopic GC in non-lymphoid organs[413–415]. Besides their engagement with autoantibody production, ELS have been implicated in the perpetuation of autoimmunity within the target organ[356,357]. While some studies questioned the importance of ELS for autoantibody production, based on the presence of ELS in seronegative (no autoantibody detection in serum) RA patients, a large body of evidence corroborated the pathogenic role of ELS in autoantibody production[413,416]. Using a human-mouse chimeric model, Humby and colleagues demonstrated that only the engraftment of ELS-positive synovial tissue from RA patients resulted in the release of human class-switched anti-citrullinated autoantibodies (the hallmark of RA), as compared to ELS-negative synovial tissues[413,417–420]. Autoreactivity towards SS-associated autoantigens, such as ribonucleoproteins Ro/SSA and La/SSB, was also detected in plasma cells which were infiltrating ELS in salivary glands of SS patients[418–420].

Notably, many of the regulatory mechanisms that ensure tolerance within GC reactions in

secondary lymphoid organs are not seen in ELS induced in autoimmune settings[143,356]. Le Pottier and colleagues demonstrated that autoreactive 9G4 B cells were not excluded from ELS found in salivary glands of SS patients[415]. Although, the mechanisms driving accumulation of autoreactive B cells in ELS are not fully understood, some reports have implicated a role for Epstein-Barr virus (EBV)[356,418,421–423]. EBV is a γ-herpesvirus which is usually acquired silently early in life and carried thereafter as an asymptomatic infection of the B lymphoid compartment[424]. ELS from patients with MS, MG, RA and SS frequently harbour latent EBV infection, where EBV-transformed B cells and plasma cells display autoreactivity to disease-specific autoantigens, such as citrullinated fibrinogen in RA and ribonucleoprotein Ro/SSA in SS[418,421–423].

Overall, the above evidence strongly supports that ELS encompass an additional and/or alternative site for autoantibody production by putative dysregulated GC reactions which perpetuate pathogenic mechanisms of autoimmune diseases. However, the prevalence of ELS with GC features varies among autoimmune diseases[356,357]. While, around 20% of ELS found in exocrine glands of SS patients have GC activity, GC are virtually absent in ELS from SLE and Autoimmune Myositis patients, suggesting that ELS account for clinical heterogeneity observed in patients with autoimmune diseases[411,425–427]. The reason underlying such variability remains unknown, although genetic predisposition and/or environmental factors shape the inflammatory response to give rise to diverse structural outcomes within individual tissue microenvironments[356,357].

4.2.3. Targeting Ectopic Lymphoid Structures

Besides their role in the pathogenesis of autoimmune diseases, ELS have been reported as useful markers of disease severity with prognosis relevance and as new therapeutic targets in autoimmunity.

In SS, the presence of ELS in salivary glands is associated with higher levels of circulating autoantibodies and systemic manifestations, as well as with the risk of subsequent mucosal-associated lymphoid tissue (MALT) lymphoma development[418,419,428–430]. The evidence for clinical relevance of ELS in RA is more controversial. While, several studies have reported that ELS are associated with increased circulating levels of inflammatory markers, autoantibody status and higher disease severity, others reports have failed to find a direct

correlation between the presence of ELS and those disease features[431,432]. In SLE, very few studies have also shown an association between the presence of renal ELS and severity of lupus nephritis[433].

Although, the results are conflicting, ELS have also been studied as predictors of response to TNF inhibitors (Infliximab) and anti-CD20 antibodies (Rituximab)[434,435]. In RA patients persistence of ELS after Infliximab treatment emerged as a negative predictor of clinical improvement[434,435]. In SS patients, treated with Rituximab, the median number of $CD20^+$ B cells within ELS in parotid gland emerged as a positive predictor of clinical improvement[436].

The advances in the understanding of the mechanisms regulating the formation, maintenance and function of ELS, and the increased appreciation of their importance in disease pathogenesis and response to treatment, have provided a strong rationale for targeting ELS in autoantibody-mediated autoimmune diseases for therapeutic purposes. So far, attempts to disrupt ELS formation using Baminercept (a fusion protein of human LTβ receptor and human IgG1) failed to demonstrate clinical efficacy in RA and SS[357]. Therapeutic approaches targeting T-B cell interactions, rather than solely disrupting ELS, might instead fine-tune the function of ectopic GC, thereby representing an attractive new strategy. However, to effectively advance the field, it is essential to stratify patients according to the presence of ELS and to refine methodologies to study mechanisms regulating ELS biology.

CHAPTER 2

AIMS OF THE THESIS

Humoral immunity is critical for the clearance of pathogens and is also the basis for protection elicited by vaccines. Failure of the immune system to enforce tolerance during humoral responses readily leads to the development of autoimmune diseases. Although, the identification of most players in the process of autoimmunity have led to development of virtuous immune-based therapies in the last decade, none is able to induce complete and sustained remission of autoimmune diseases. Moreover, very few immune-based therapies target endogenous regulatory mechanisms, especially those used to survey the emergence of self-reactive B cells within GCs.

Tfr cells regulate GC reactions presumably patrolling the development of B cell harbouring high affinity self-reactive antibodies. While many features of Tfr cell biology remain elusive, targeting Tfr cells or Tfr cell-dependent regulatory mechanisms could become a novel approach to treatment of autoimmune diseases. This concept relies on the assumption that human blood $CXCR5^{+}Foxp3^{+}$ T cells are circulating Tfr cells, as these are the putative Tfr cells easily accessible in humans.

The overall aim of this thesis was to investigate the **biology of Tfr cells in human adaptive immunity and autoimmunity**, by addressing two main questions:

1. **Do blood $CXCR5^{+}Foxp3^{+}$ Treg cells constitute circulating counterparts of germinal centre Tfr cells?**

   Following the identification of Tfr cells as key regulators of humoral responses in murine secondary lymphoid organs, many studies categorically assumed $CXCR5^{+}Foxp3^{+}$ T cells in human blood are circulating Tfr cells (as circulating $CXCR5^{+}$ T cells were demonstrated to be circulating counterparts of tissue Tfh cells with memory properties[52,54–56]). However, the formal demonstration of a specialized subset of human blood Treg cells engaged with regulation of humoral responses was never achieved. Besides the limited access to human secondary lymphoid tissues, Foxp3 and CXCR5 upregulation by human non-regulatory T cells undergoing activation creates a further challenge on the assumption that peripheral blood $CXCR5^{+}Foxp3^{+}$ T cells are indeed *bona fide* circulating Tfr cells[232–235,326].

The first part of the results describes several experiments designed to investigate whether human blood CXCR5$^+$Foxp3$^+$ T cells are indeed circulating counterparts of *bona fide* Tfr cells. We analysed the phenotype of CXCR5$^+$Foxp3$^+$ T cells in different human tissues, namely, blood, cord blood, tonsils and thymus. In addition, we optimized and performed various *in vitro* assays to directly address the function of this cell population. Finally, we used blood samples from B cell-deficient patients and from a prospective cohort of healthy volunteers undergoing seasonal influenza vaccination to demonstrate the intricate relationship between blood CXCR5$^+$Foxp3$^+$ T cells and GC responses.

We found that CXCR5$^+$Foxp3$^+$ T cells are suppressive Foxp3$^+$ Treg cells with migratory capacity toward B cell follicles, thus comprising a circulatory compartment of *bona fide* Tfr cells. We also found that CXCR5$^+$Foxp3$^+$ T cells increase after the induction of GC responses by vaccination and are absent from neonatal cord blood (an immune setting in which GC reactions do not take place), confirming their relationship with humoral responses in secondary lymphoid organs. However, as these cells gain access to circulation prior to B-cell interaction, they are immature and not yet proficient in regulating humoral responses. Therefore, blood Tfr cells are not fully competent Tfr cells.

2. Do blood CXCR5$^+$Foxp3$^+$ Treg cells constitute a novel biomarker for autoantibody-mediated systemic autoimmune diseases?

After the demonstration that blood Tfr cells arise following humoral responses, we wondered whether this Treg cell subset is altered in autoimmune diseases, specifically those with autoantibody production in ectopic (tertiary) lymphoid structures[356,357]. We hypothesized that blood Tfr cells would be increased in patients with autoimmune diseases, rather than decreased, as blood Tfr cells do not represent the humoral suppressive potential of the immune system.

Sjögren syndrome (SS) is a systemic autoimmune disease characterized by ectopic lymphoid neogenesis in exocrine glands where autoantibodies are produced due to dysregulated T – B cell interactions[415,420,437–439].

Taking advantage of paired blood and tissue (salivary gland biopsies) samples from patients referred for clinical evaluation of SS we addressed whether blood Tfr cells could be used to identify SS patient with autoantibody production and ectopic lymphoid activity. These results are described in the second part of the thesis results.

We found that blood Tfr cells, and specifically the Tfr/Tfh ratio, are increased in SS patients. Notably, patients with ectopic lymphoid activity have the highest level of blood Tfr/Tfh ratio. These findings led us to propose blood Tfr/Tfh ratio as a novel biomarker of ectopic lymphoid activity in SS, a finding with potential clinical implications.

CHAPTER 3

MATERIALS AND METHODS

1. Patient Recruitment and Human Samples

1.1. Healthy Donor's Blood Samples

Fresh peripheral blood samples were collected from 42 adult healthy volunteers (recruited from a Lisbon Health Centre: *USF do Parque*), with no known medical condition, no vaccination or infection in the past two weeks, and no chronic medication. Fresh buffy-coats (blood collection in less than 24 hours) from *Instituto Português do Sangue e da Transplantação, Lisboa, Portugal* were used for *in vitro* functional assays. All blood samples were collected in EDTA coated tubes.

For vaccination studies, we used 32 adult healthy volunteers recruited from the Cambridge BioResource as part of the vaccination study during the 2014 – 2015 winter season. Participants were excluded if they have had a previous adverse reaction to any vaccination, a known allergy to any components of the vaccine, were taking immune modulating medication, and women who were pregnant or breastfeeding. Participants were administered the inactivated influenza vaccine (split virion) BP vaccine (Sanofi Pasteur) by intramuscular injection in the right deltoid. Blood samples were collected in EDTA coated tubes on the day of vaccination (prior to administration of the vaccine) and 7 days following vaccination. These data were obtained as part of a collaborative work with Michell A Linterman's laboratory at Babraham Institute, Cambridge, UK.

1.2. B-cell Deficient Patients

Fresh blood samples were collected from 5 adult X-linked Agammaglobulinemia (BTK-deficient) patients before schedule treatment with intravenous immunoglobulin from *Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, Lisboa, Portugal*. Blood samples were collected in EDTA coated tubes.

1.3. Children Blood and Tissue Samples

Thymus tissue was collected from 4 children submitted to cardiac surgery due to congenital heart disease who were otherwise healthy, under a collaborative work with Ana E Sousa's laboratory, *Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de*

Lisboa, Lisboa, Portugal and *Centro Hospitalar Lisboa Ocidental, Hospital de Santa Cruz, Lisboa, Portugal.*

Tonsils and fresh peripheral blood samples were collected from 6 healthy children submitted to tonsillectomy due to tonsil hypertrophy at *Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, Lisboa, Portugal*. Children with any clinical condition, under any drug treatment or submitted to tonsillectomy due to chronic tonsillitis were excluded.

Umbilical cord blood samples were collected from 3 healthy pregnancies during delivery, at *Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, Lisboa, Portugal*. Blood samples were collected in EDTA coated tubes.

1.4. Sjögren Syndrome Patients

We recruited 56 patients referred to the Rheumatology and Metabolic Bone Diseases Department of *Centro Hospitalar Lisboa Norte, Lisboa, Portugal* for minor salivary gland (MSG) biopsy due to clinical suspicion of SS. Fresh blood samples were collected on the day of MSG biopsy. MSG tissue was divided for routine diagnostic purposes and for flow cytometry and microscopy. We excluded patients treated with biologic drugs, disease modifying anti-rheumatic drugs (DMARDs) other than hydroxychloroquine or more that 7.5 mg per day of prednisolone equivalent, diagnosed with an infectious disease or who had received any vaccine in the previous month, and those with exclusion criteria for SS (**Figure 4**)[440]. Patients were classified as having primary SS if they fulfilled American European Consensus Group (AECG) 2002 classification criteria[440], whereas those with sicca symptoms and no evidence of an inflammatory rheumatic disease were classified as non-Sjögren sicca syndrome and used as appropriate controls (**Figure 4**). Patients with secondary SS or with clinical diagnosis of primary SS but not fulfilling AECG criteria were excluded from analysis to standardize populations. Disease activity was evaluated by the European League Against Rheumatism SS disease activity score (ESSDAI)[441]. Routine serum autoantibodies, c-reactive protein (mg/dL), erythrocyte sedimentation rate (mm) and serum electrophoresis gamma-fraction (g/dL) levels closest to MSG biopsy were used. Blood samples were collected in EDTA coated tubes.

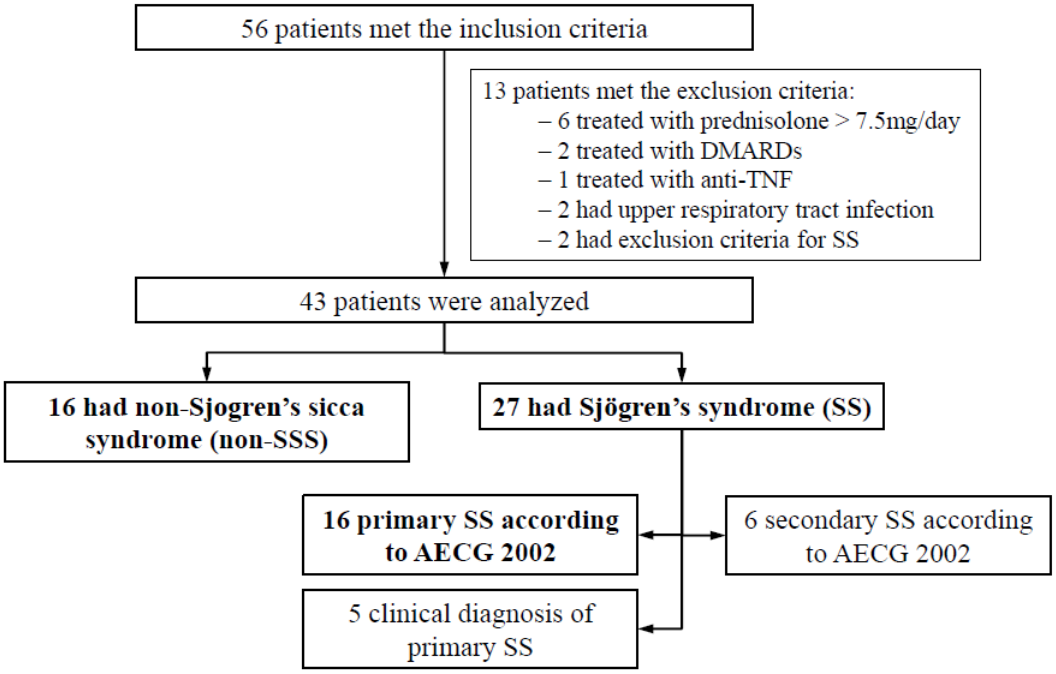


Figure 4: Flowchart of Sjögren syndrome patient recruitment and selection.
DMARDs, disease modifying anti-rheumatic drugs; TNF, tumor necrosis factor; AECG, American-European Consensus Group criteria for Sjögren's syndrome.

1.5. Ethical Issues

Studies were approved by the Lisbon Academic Medical Center Ethics Committee (reference number 505/14). Informed consent was obtained from all adult volunteers, parents or legal guardians. The influenza vaccination study protocol was approved by the Health Research Authority, National Research Ethics Service Committee South Central, Hampshire A, UK (REC reference:14/SC/1077).

2. Cell Isolation and Flow Cytometry

PBMCs were isolated from blood samples by Ficoll-gradient medium (Histopaque-1077, Sigma-Aldrich) using SepMate tubes (StemCell Technologies). Lymphocytes from tonsils and thymocytes were also isolated by Ficoll-gradient medium after mechanical disruption. Before cell sorting, PBMCs from Buffy-coats were enriched for $CD4^+$ T cells using MojoSort Human CD4 T Cell Isolation Kit (BioLegend). The $CD4^+$ fraction was used for cell sorting of $CD4^+$ T cell subsets. The $CD4^-$ fraction was used for cell sorting of naïve B cells (supplementary Figure 1a for sorting strategy).

To study human MSG biopsies, we first optimized the experimental protocol to ensure that enzymatic digestion of tissue had negligible impact on cellular markers. A cell suspension was prepared from salivary gland tissue for flow cytometry analysis. The salivary gland was cut into small fragments and incubated at 37ºC with Liberase TM 0.1 mg/mL plus DNAse I 0.1 mg/mL in RPMI medium for 20 minutes. After washing, fragments were incubated again for 10 minutes with the same enzyme solution. After washing, fragments were vigorously pipetted and filtered to obtain a homogenous cell suspension.

For flow cytometry cells were stained with anti-Bcl-6 (K112-91, BD Biosciences), anti-CCR6 (G034E3, BioLegend), anti-CCR7 (#150503, R&D Systems), anti-CD127 (eBioRDr5, eBioscience), anti-CD19 (HIB19, BioLegend), anti-CD25 (BC96, eBioscience), anti-CD27 (LG.7F9, eBioscience), anti-CD3 (OKT3, eBioscience), anti-CD31 (WM-59, eBioscience), anti-CD38 (HB-7, BioLegend), anti-CD4 (OKT4, BioLegend), anti-CD45 (HI30, BioLegend), anti-CD45RA (HI100, eBioscience), anti-CD45RO (UCHL1, BioLegend), anti-CD57 (HNK-1, BioLegend), anti-CD62L (DREG-56, BioLegend), anti-CD69 (FN30, BioLegend), anti-CD8 (RPA-T8, eBioscience), anti-CTLA-A (L3D10, BioLegend), anti-CXCR3 (G025H7, BioLegend), anti-CXCR5 (J252D4, BioLegend), anti-Foxp3 (PCH101, eBioscience), anti-HLA-DR (G46-6, BD Biosciences), anti-ICOS (C398.4A, BioLegend), anti-IgD (IA6-2, BioLegend), anti-Ki67 (Ki-67, BioLegend), anti-PD-1 (EH12.2H7, BioLegend). For Bcl-6, CTLA-4, Foxp3, and Ki67 intracellular staining, Foxp3 Fix/Perm Kit (eBioscience) was used according to manufacturer's instructions. For cell viability staining, Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used. Cell Trace Violet Cell Proliferation Kit (Life Technologies) was used for cell proliferation assessment. Cell sorting was performed in Aria IIu and Aria III instruments (BD Biosciences). Flow cytometry analysis was performed in a LSR Fortessa instrument (BD Biosciences) and further analyzed with FlowJo v10 software (TreeStar).

3. Functional Assays

3.1. Suppression Assays and *in vitro* Cultures

For *in vitro* suppression assays 25 x 10^3 $CXCR5^-CD25^-CD127^+CD4^+$ conventional T cells or 25 x 10^3 $CXCR5^+CD25^-CD127^+CD4^+$ Tfh cells were plated with $CXCR5^-CD25^+CD127^-CD4^+$ Tregs cells or $CXCR5^+CD25^+CD127^-CD4^+$ Tregs cells in 1:1 ratio. Cells were

cultured with 1 μg/mL anti-CD3 (OKT3, eBioscience) in the presence of 10^5 irradiated (2500 rad) allo-PBMCs. After 5 days, cells were harvested and responder cells were analyzed for CTV dilution by flow cytometry. For TCR stimulation assays 25 x 10^3 $CXCR5^-CD25^+CD127^-CD4^+$ Tregs cells and $CXCR5^+CD25^+CD127^-CD4^+$ Tregs were plated with 1 μL/well of anti-CD3/anti-CD28 MACSiBead particles (T Cell Activation Kit, Miltenyi Biotec).

For co-culture *in vitro* suppression assays 25 x 10^3 $CXCR5^+CD25^-CD127^+CD4^+$ Tfh cells were plated with $CXCR5^-CD25^+CD127^-CD4^+$ Tregs cells or $CXCR5^+CD25^+CD127^-CD4^+$ Tregs cells in 1:1 ratio, in presence of 30 x 10^3 $CD27^-IgD^+CD19^+$ naïve B cells. Cells were cultured with 1 μg/mL SEB (Sigma-Aldrich). After 5 days, responder Tfh cells were analyzed for CTV dilution, B cells for CD38 upregulation, and Tregs for follicular and activation markers.

For immunoglobulin measurement 25 x 10^3 $CXCR5^+CD25^-CD127^+CD4^+$ Tfh cells were plated with $CXCR5^-CD25^+CD127^-CD4^+$ Tregs cells or $CXCR5^+CD25^+CD127^-CD4^+$ Tregs cells in 1:1 ratio, in presence of 30 x 10^3 $CD27^-IgD^+CD19^+$ naïve B cells. Cells were cultured with 1 μg/mL SEB (Sigma-Aldrich) + 10 ng/mL SEA (Toxin Technology) + 10 ng/mL SEE (Toxin Technology) + 10 ng/mL TSST-1 (Toxin Technology). After 10 days, supernatants were collected and immunoglobulin concentration determined by ELISA.

Cultures were performed in U-shape 96 wells plates in RPMI medium (RPMI 1640, Life Technologies) supplemented with 10% heat-inactivated Fetal Bovine Serum (Life Technologies), 1% HEPES (Sigma-Aldrich), 1% Sodium Pyruvate (Life Technologies), 1% PenStrep (Life Technologies), and 0.05% Gentamicin (Life Technologies) and in 37ºC, 5% CO_2 incubator conditions.

3.2. ELISA

IgA, IgM, and total IgG concentration were determined in supernatants from T-B co-culture (as described above) by ELISA using Human ELISA Ready Set Go Kit, according to the manufacturer's instructions (eBioscience).

3.3. Migration Assays

For *in vitro* chemotaxis assays 75 x 10^3 $CXCR5^-CD25^-CD127^+CD4^+$ conventional T cells, $CXCR5^+CD25^-CD127^+CD4^+$ Tfh cells, $CXCR5^-CD25^+CD127^-CD4^+$ Tregs cells and $CXCR5^+CD25^+CD127^-CD4^+$ Tregs cells, sorted from buffy-coats, were loaded on top wells of HTS Transwell 96-well permeable supports (5μm pore size) (Corning). Plain RPMI medium (RPMI 1640, Life Technologies) or medium supplemented with 0.2 μg/mL CXCL13 (Peprotech) was added to the bottom wells of the plate. After 4 hours of incubation (37ºC, 5% CO_2), filters were removed and cells that migrated to the lower chamber were counted in a LSR Fortessa instrument (BD Biosciences) and further analyzed with FlowJo v10 software (TreeStar). Chemotaxis index was calculated as the ratio of cells migrating toward CXCL13 and cells randomly migrating.

4. Real-time RT-PCR

Total RNA was extracted, and reverse transcribed from FACS-sorted $CXCR5^-CD25^-CD127^+CD4^+$ conventional T cells, $CXCR5^+CD25^-CD127^+CD4^+$ Tfh cells, $CXCR5^-CD25^+CD127^-CD4^+$ Tregs cells and $CXCR5^+CD25^+CD127^-CD4^+$ Tregs from buffy-coats, using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. cDNA was generated using SuperScript III reverse transcriptase (Life Technologies), according to manufacturer instructions. Real-time PCR was set up with Power SYBR Green PCR Master Mix (Applied Biosystems) and performed on ViiA 7 Real-Time PCR System (Applied Biosystems), according to manufacturer's instructions. The expression of each gene was normalized to housekeeping genes (B2M, ACTB or G6PD) and calculated by change-in-threshold method (ΔC_T), using QuantStudio Real-Time PCR software v1.1 (Applied Biosystems). The following primers (Invitrogen) were used: Foxp3, 5'-GCAAATGGTGTCTGCAAGTG-3' (forward) and 5'-GCCCTTCTCATCCAGAAGAT-3' (reverse); CXCR5, 5'-CTGGAAATGGACCTCGAGAA-3' (forward) and 5'-GCAGGGCAGAGATGATTTTC-3' (reverse); Bcl-6, 5'-TTCCGCTACAAGGGCAAC-3' (forward) and 5'-CGAGTGTGGGTTTTCAGGTT-3' (reverse); B2M, 5'-TATGCCTGCCGTGTGAACCAT-3' (forward) and 5'-CGGCATCTTCAAACCTCCATG-3' (reverse); ACTB, 5'-CTCTTCCAGCCTTCCTTCCT-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3' (reverse); G6PD, 5'-CCAAGCCCATCCCCTATATT-3' (forward) and 5'-CCACTTGTAGGTGCCCTCAT-3' (reverse).

5. Microscopy

5.1. Human Tonsils

After paraffin removal and antigen retrieval by heat (HIER pH 9, Leica Biosystems) 3 µm sections of formalin-fixed paraffin-embedded human tonsil were stained with anti-human CXCR5-Alexa-Fluor 488 (J252D4, BioLegend), anti-human CD4 (SP35, CellMarque) and anti-human Foxp3 (PCH101, eBioscience) primary antibodies. Alexa-Fluor 488 (anti-mouse), Alexa-Fluor 546 (anti-rabbit) and Alexa-Fluor (anti-Rat) were used as secondary antibodies, respectively. DAPI was used as nuclei counterstaining. Images were acquired with ZEN 2012 software on a Zeiss LSM 710 confocal point-scanning microscope (Carl Zeiss, Oberkochen, Germany) using a dry plan-apochromat 20x objective (200x magnification) and with a numerical aperture of 0.80. Images were further analyzed using Image-J FiJi software.

5.2. Minor Salivary Gland Biopsies

MSG were fixed in 10% buffered formaldehyde, embedded in paraffin wax and sectioned into 3 µm sequential sections. Sections were stained after deparaffinisation, pretreatement with Ultra CC1 (Ventana Medical Systems, USA), antigen retrieval and endogenous peroxidase blocking using Benchmark machine. Sections were immunohistochemically stained with anti-human CD20 (L26, DAKO), anti-human CXCR5-Alexa-Fluor 488 (J252D4, BioLegend), and anti-human Foxp3 (236A/E7, eBiosciences). The sections were then treated with a peroxidase-labelled secondary antibody and visualized with the chromogen DAB solution and/or universal AP Red. Hematoxylin was used as counterstaining. Images were acquired with NanoZoomer-SQ (Hamamatsu, Japan) using a 20x objective with a numerical aperture of 0.75. Images were further analyzed using NDP.view software. Number of CXCR5 and Foxp3 double positive cells were counted manually. Routine hematoxylin and eosin staining was used to define histological diagnosis in MSG: Normal, section with no pathological findings; LI, sections with unspecific lymphocytic infiltration; and focal sialadenitis (FSA), sections with ≥1 dense aggregate of ≥50 lymphocytes per 4 mm^2 of tissue located in perivascular or periductal locations[442].

6. Statistical analysis

Unpaired Student T-test, one-way ANOVA with post-test Turkey's multiple comparison, and two-way ANOVA with post-test Bonferroni's multiple comparison were used as described. Normality of data was assessed by Shapiro-Wilk test. Variance across groups was assessed with Levene's and Brown–Forsythe's tests and, where different, Welch correction of T-test or Mann Whitney U test were preferred. Pearson correlation and linear regression were also conducted for some data. For categorical variables Fisher's exact test was used. Logistic regression and receiver operating characteristic (ROC) analysis were conducted to study the association of Tfr/Tfh ratio with SS and FSA and the cut-off with the best discriminative value was determined. Results are presented as mean ± SD. P values of less than 0.05 were considered statistically significant. GraphPad Prism v5 and Stata v.12.1 were used for statistical and graphical analysis. Correlation heatmaps were computed in R studio v3.1.2.

CHAPTER 4

RESULTS

Human Blood CXCR5$^+$Foxp3$^+$ Treg Cells Are Immature Tfr Cells Not Fully Licensed with Humoral Suppressive Function

Introduction

GC responses are crucial for the generation of high affinity antibodies during T-dependent immune responses. Within the GC resides a specialized subset of CD4$^+$ T cells – the Tfh cells – which are essential for GC development and function[63,443]. It is now clear that Tfh cells play a central role in productive vaccine responses, while defects in their formation or function can contribute to immunodeficiency or autoimmunity[390,392]. More recently, the discovery of Tfr cells, a subset of suppressive regulatory T cells that participate in the GC, added an additional layer of complexity in the biology of GC responses[257,256,254,255].

Tfr cells, generally defined by Bcl-6$^+$CXCR5$^+$PD-1$^+$ICOS$^+$Foxp3$^+$, are a distinct subset of thymic Foxp3$^+$ Treg cells present in lymphoid tissues. Like the Tfh cell differentiation pathway, Tfr cell-commitment require both dendritic cell and B cell interactions, as well as CD28, SAP, ICOS, and PD-1 signaling[258,260,255]. A tight balance between expression of transcription factors Bcl-6 and Blimp-1 regulates the differentiation of Tfr cells[255]. Tfr cells have specialized functions in controlling the magnitude of GC responses and in limiting the outgrowth of non-antigen-specific B cell clones[256,255]. However, the precise mechanisms of Tfr cell suppression remain elusive, although CTLA-4 and regulation of metabolic pathways seem to play a key role[259,269,272].

Although, Tfh and Tfr cells are characterized by their location in lymphoid tissues an increasing number of studies have described putative circulating counterparts of these cells in peripheral blood. This is particularly relevant for studying the biology of these cells in humans, as access to secondary lymphoid tissues can be limiting. Human blood CXCR5$^+$ T cells have been established as memory Tfh-like cells, based on their ability to recapitulate *bona fide* Tfh cell functions: human blood CXCR5$^+$ T cells can promote plasmablast differentiation, AID expression and class switch recombination by naïve B cells. However, they are phenotypically distinct from tissue Tfh cells and do not express the transcriptional repressor Bcl-6[52,55,56]. Furthermore, an immunization leading to GC and antibody responses

correlates with an increase in the frequency of circulating ICOS$^+$ Tfh cells, suggesting that they indicate ongoing Tfh cell responses in secondary lymphoid tissues[52,54,55,120,123]. Human circulating Tfh cells comprise a heterogeneous population concerning their phenotype and the quality of help they provide to B cells[52,54].

In mice, CXCR5$^+$Foxp3$^+$ Tfr-like cells were found in peripheral blood after immunization, and shown to represent a circulating counterpart of tissue Tfr cells[258,260].

Although, CXCR5-expressing Treg cells and GC Foxp3-expressing T cells have been found in humans[254,323,325], so far, no study have addressed the biological significance of these putative circulating Tfr-like cells in humans. Human tonsil CD25$^+$CD69$^-$ T cells have been shown to directly suppress B cell responses, but the relationship of these putative Treg cells to Bcl-6$^+$CXCR5$^+$PD-1$^+$ICOS$^+$Foxp3$^+$ Tfr cells is unclear[444,445]. Peripheral blood CXCR5$^+$ Treg cells are being studied as circulating Tfr cells in many different human diseases, despite the biological relevance of these cells being unclear[329,333,338,341,446]. Additionally, Foxp3 upregulation by non-regulatory human T cells and transient CXCR5 expression by T cells undergoing activation challenge the assumption that peripheral blood CXCR5$^+$ Treg cells are indeed *bona fide* circulating Tfr cells[235,326].

Here, we found that human blood Tfr cells, defined as CXCR5$^+$Foxp3$^+$ T cells, are generated in peripheral lymphoid tissues as humoral immune responses are established. However, in contrast to tissue Tfr cells and conventional CXCR5$^-$ Treg cells, circulating Tfr cells have a naïve-like phenotype. Our data demonstrate that blood Tfr cells are generated following the initial steps that lead to GC responses being distinct from tissue Tfr cells.

Results

Blood and tissue Tfr cells present different follicular and regulatory markers

To test whether CXCR5$^+$Foxp3$^+$ Tfr cells in human peripheral blood are circulating counterparts of tissue Tfr cells, we studied peripheral blood from a cohort of 42 healthy volunteers between 22 and 92 years old (mean age 46.76 ± 18.14 years old, 30 females and

12 males). We found that CXCR5 was expressed by 18.57 ± 6.55% of total Tregs (defined as $CD4^+CD25^+Foxp3^+$ T cells) (**Figure 5A**). The frequency and number of $CXCR5^+Foxp3^+$ Tfr cells did not change with aging (**Figure 5B, C**).

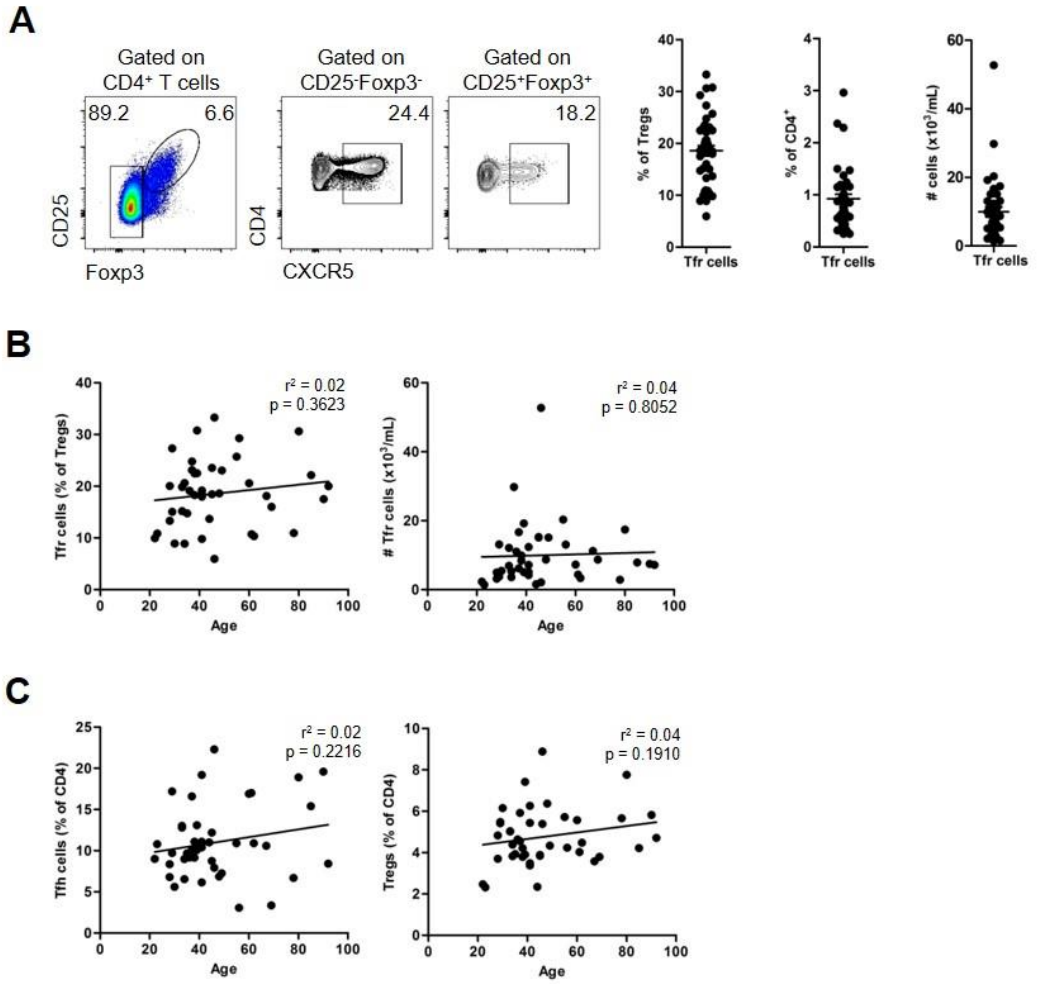


Figure 5: Identification of blood Tfr cells.
(A) $CXCR5^+$ Tfr cells constitute 18.57 ± 6.55% of Tregs (left) and 0.93 ± 0.56% of total $CD4^+$ T cells (middle), representing 9985 ± 9043 cells per mL of blood (right) (n = 42, adult healthy donors). **(B)** Variation of blood Tfr cells frequency (left) and absolute number per ml of blood (right) accordingly to age (age range: 22 – 92 years old). (n = 42, linear regression). **(C)** Variation of blood Tfh cells (left) and total Tregs (right) frequency accordingly to age (age range: 22 – 92 years old) (n = 42, linear regression). Error bars represent SEM.

As CXCR5 is used to identify human circulating Tfh cells, we compared the phenotype of circulating $CXCR5^+Foxp3^+$ Tfr cells with that of circulating Tfh cells and $CXCR5^-$ conventional Treg cells. Peripheral blood $CXCR5^+Foxp3^+$ T cells share characteristics with both circulating Tfh cells and $CXCR5^-$ Treg cells (**Figure 6A-C**). Bcl-6 expression was not

detected in any population by real-time PCR (**Figure 6D)**, consistently with previous reports showing that blood Tfh cells do not express Bcl-6[53–56,120].

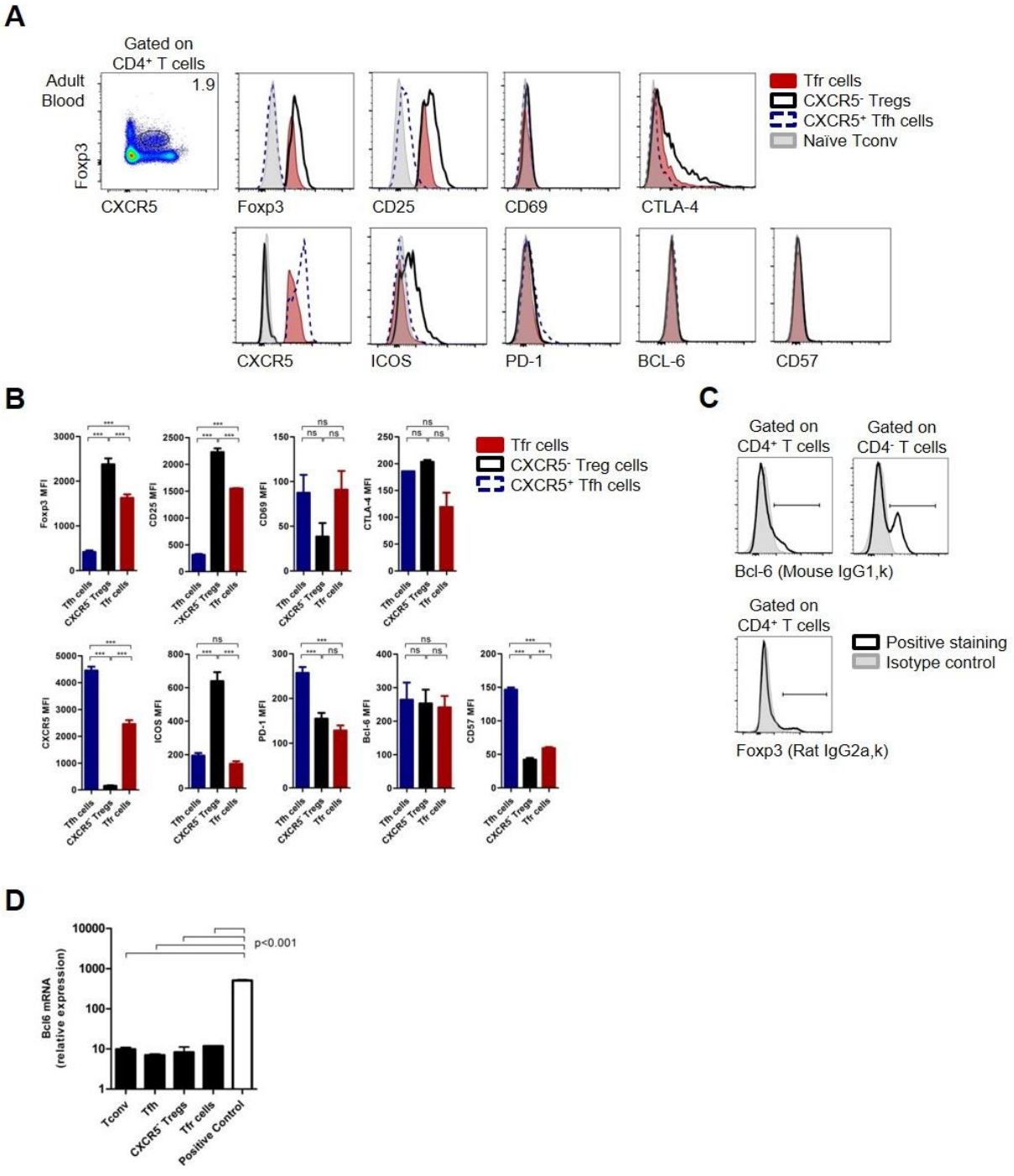


Figure 6: Blood Tfr cells show expression of follicular and regulatory markers.
(A) Expression of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1 Bcl-6, and CD57 by Tfh cells (blue), CXCR5$^-$ Tregs (black) and Tfr cells (red), in adult. Naïve CD4$^+$ T cells were used as control (gray). Representative plots from 42 healthy volunteers. **(B)** MFI of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1 Bcl-6, and CD57 by Tfh cells (blue), CXCR5$^-$ Tregs (black) and Tfr cells (red) in adult blood (**p<0.01, ***p<0.001, n = 6, one-way ANOVA with post-test Turkey's multiple comparison) **(C)** Isotype controls for intracellular Bcl-6 and Foxp3 staining by

flow cytometry. **(D)** Relative expression of Bcl-6 by sorted Tconv, Tfh cells, CXCR5- Tregs and Tfr cells from blood, by real-time RT-PCR. Gene expression normalized to housekeeping genes (B2M, G6PD and ACTB) (n = 2, each with technical duplicates, Student t-test). $CD38^{hi}IgD^{-}$ germinal center B cells sorted from human tonsils were used as positive control. Error bars represent SEM.

Taking advantage of routine tonsillectomies performed due to tonsil hypertrophy in otherwise healthy children, we compared the cell phenotype of paired blood and tissue samples from the same child (**Figure 7A, B**).

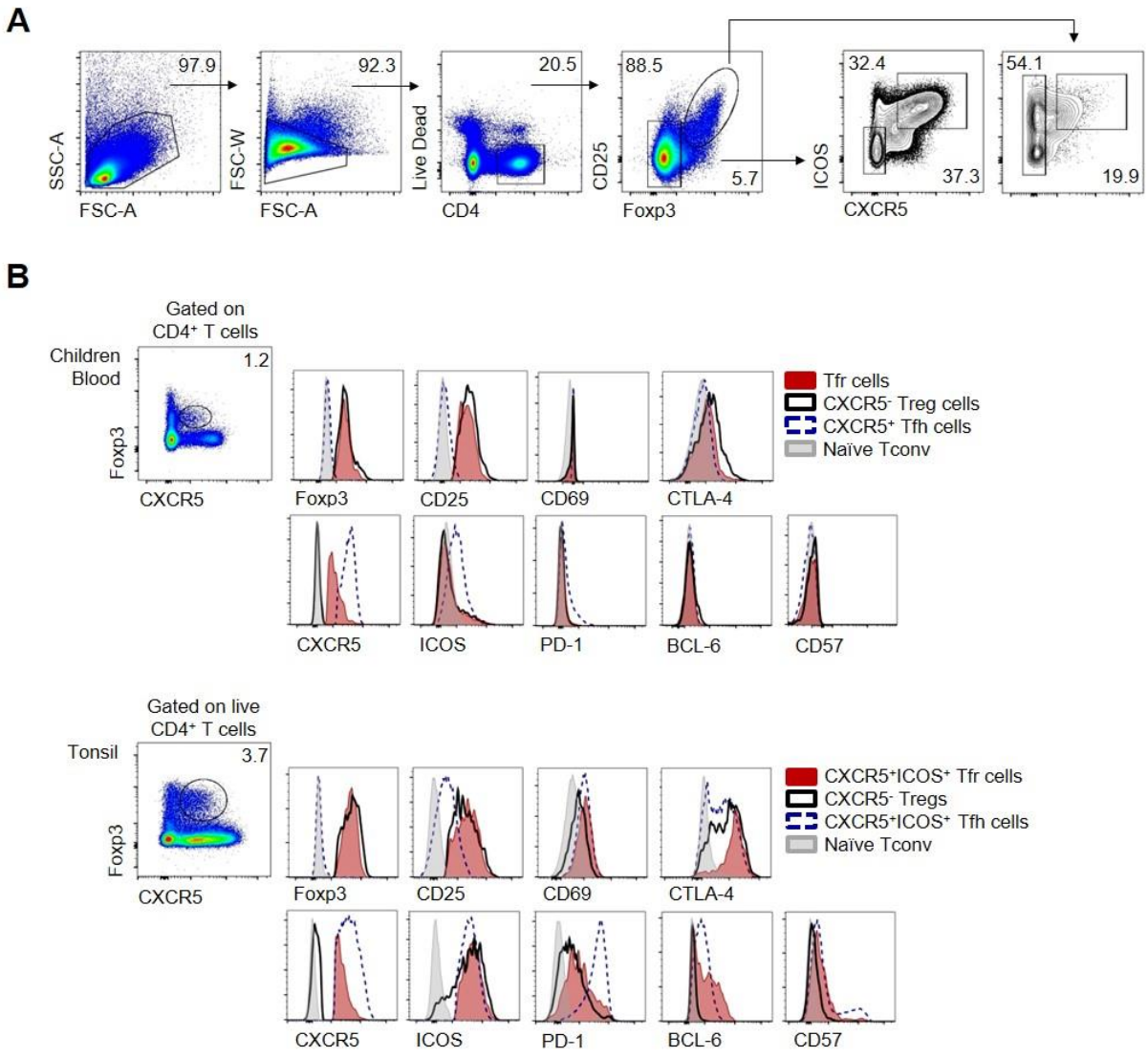


Figure 7: Blood Tfr cells are distinct from their tissue counterparts.
(A) Gating strategy for identification and analysis of Tconv, Tfh cells, $CXCR5^{-}$ Tregs and $CXCR5^{+}$ Tregs in human tonsils. **(B)** Expression of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1 Bcl-6, and CD57 by Tfh cells (blue), $CXCR5^{-}$ Tregs (black) and Tfr cells (red), in children blood (top rows) and in tonsils (bottom rows). Naïve $CD4^{+}$ T cells were used as control (gray). Representative plots from 6 healthy children.

We found that circulating Tfh cells were phenotypically distinct from their tissue counterparts, in line with previous reports, especially regarding their PD-1, ICOS and Bcl-6 expression (**Figure 7B**)[52,56]. In an analogous way, circulating $CXCR5^{+}Foxp3^{+}$ Tfr cells were also $ICOS^{-}PD\text{-}1^{-}Bcl\text{-}6^{-}CD57^{-}$, and consequently distinct from tonsil Tfr cells (**Figure 7B**). Our results are consistent with murine studies showing that blood and tissue Tfr cells are phenotypically distinct[258]. Notably, ICOS was not differentially expressed by Treg cells and Tfr cells in tonsils (**Figure 7B**). We also confirmed that tissue $CXCR5^{+}Foxp3^{+}$ T cells are localized within GCs, therefore, corresponding to Tfr cells (**Figure 8A, B)**. Curiously, we observed different Tfr/Tfh ratios in the blood and tonsils (**Figure 8C**).

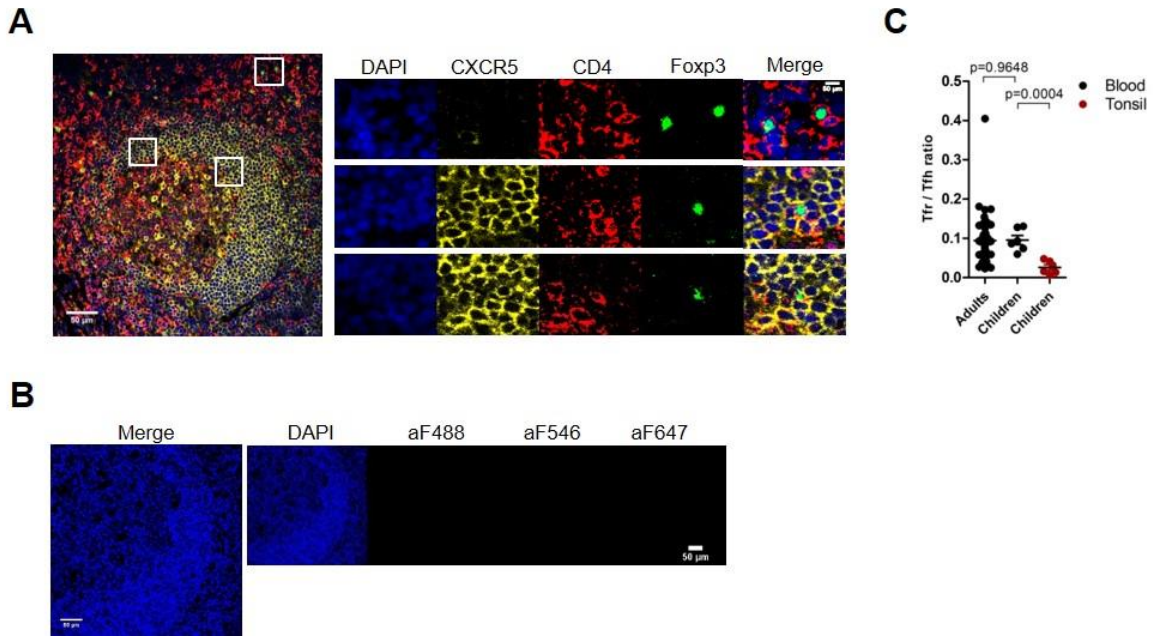


Figure 8: $CXCR5^{+}Foxp3^{+}$ T cells are localized within GCs in human secondary lymphoid organs.

(A) Immunofluorescence microscopy of formalin-fixed paraffin-embedded human tonsils stained for DAPI (blue), CXCR5 (yellow), CD4 (red) and Foxp3 (green). Top, middle and bottom outlined areas indicate top, middle and bottom enlarged areas on the right, respectively. Data are representative of tonsil sections from 5 healthy children. **(B)** Negative control for immunofluorescence microscopy, merge (left) and composite of the four immunofluorescence channels (right). After paraffin removal and antigen retrieval by heat sections of formalin-fixed paraffin-embedded human tonsil were stained with Alexa-Fluor 488 (anti-mouse), Alexa-Fluor 546 (anti-rabbit) and Alexa-Fluor (anti-Rat) secondary antibodies, without primary antibodies. DAPI was used as nuclei counterstaining. Unspecific binding of secondary antibodies and cross-reactivity between secondary antibodies were excluded. **(C)** Blood Tfr/Tfh ratio in adult blood, children blood (tonsil donors) and in tissues (tonsils). Black and red dots represent blood and tonsil results, respectively (n = 42 for adults and n = 6 for children, Student t-test). Error bars represent SEM.

CXCR5^{+}Foxp3^{+} Tfr cells are a distinct subset of suppressive Foxp3^{+} T cells

It has been described that CXCR5 expression can transiently occur upon human T cell activation[49,326,327]. Moreover, human T cells can also transiently express Foxp3 upon *in vitro* TCR stimulation in a TGF-β dependent manner[235,328]. To address whether *ex vivo* CXCR5^{+}Foxp3^{+} Tfr cells were *bona fide* regulatory cells, we sorted that cell population, as well as CXCR5^{-} conventional Treg cells (**Figure 9A,B**).

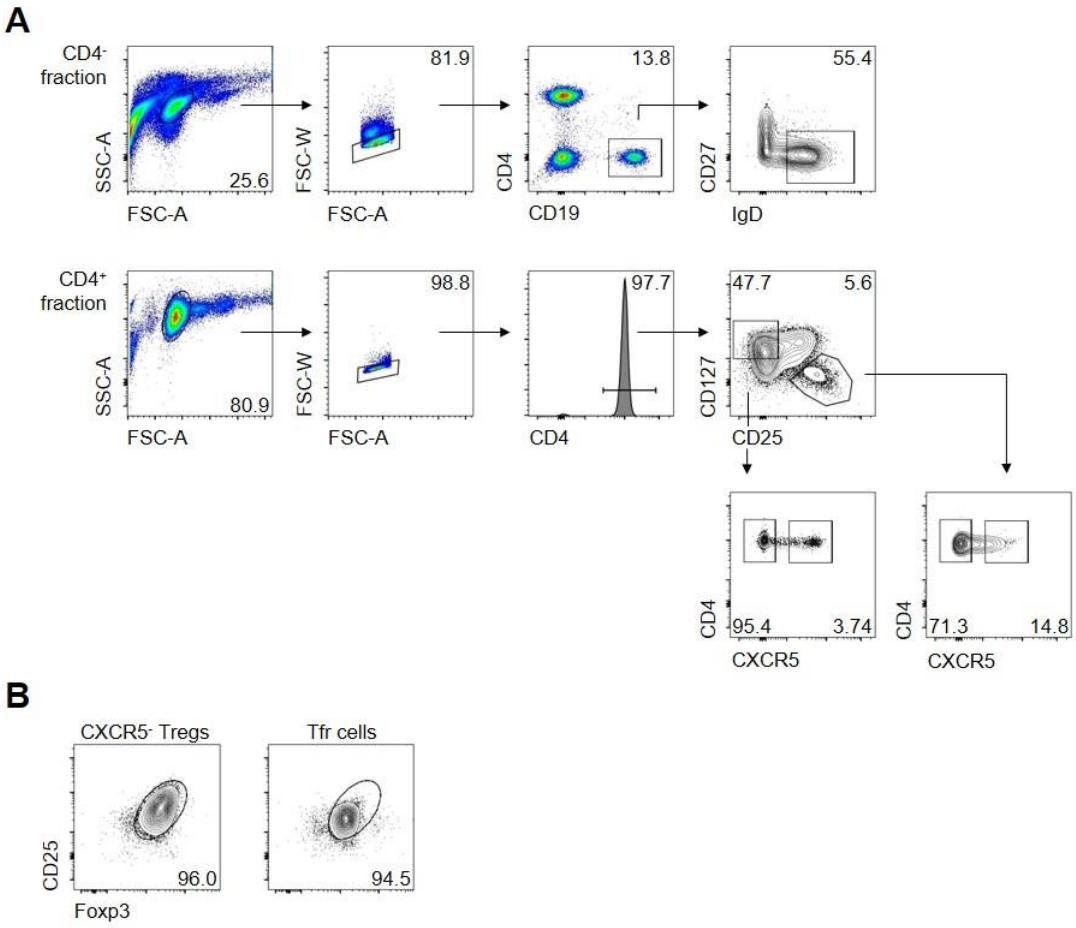


Figure 9: Sorting strategy for human blood Tconv, Tfh, CXCR5^{-} Treg, Tfr cells, and naïve B cells.
(A) Sorting strategy for FACS-sort of CXCR5^{+}CD25^{-}CD127^{+}CD4^{+} Tfh cell, CXCR5^{-}CD25^{-}CD127^{+}CD4^{+} Tconv cell, CXCR5^{+}CD25^{+}CD127^{-}CD4^{+} Tfr cells, CXCR5^{-}CD25^{+}CD127^{-}CD4^{+} Treg cells, and CD27^{-}IgD^{+}CD19^{+} naïve B cell populations from peripheral blood (buffy-coats). **(B)** Purity of FACS-sorted CXCR5^{+}CD25^{+}CD127^{-}CD4^{+} Tfr cells, CXCR5^{-}CD25^{+}CD127^{-}CD4^{+} Treg cells.

We cultured CXCR5^{-} Treg and Tfr cells with CTV-labelled conventional T cells. Proliferation of responder cells was analysed after 5 days of soluble αCD3 stimulation

(**Figure 10A**). Blood CXCR5^{+}Foxp3^{+} T cells significantly reduced conventional T cell proliferation (**Figure 10B, C**), clearly demonstrating their regulatory function.

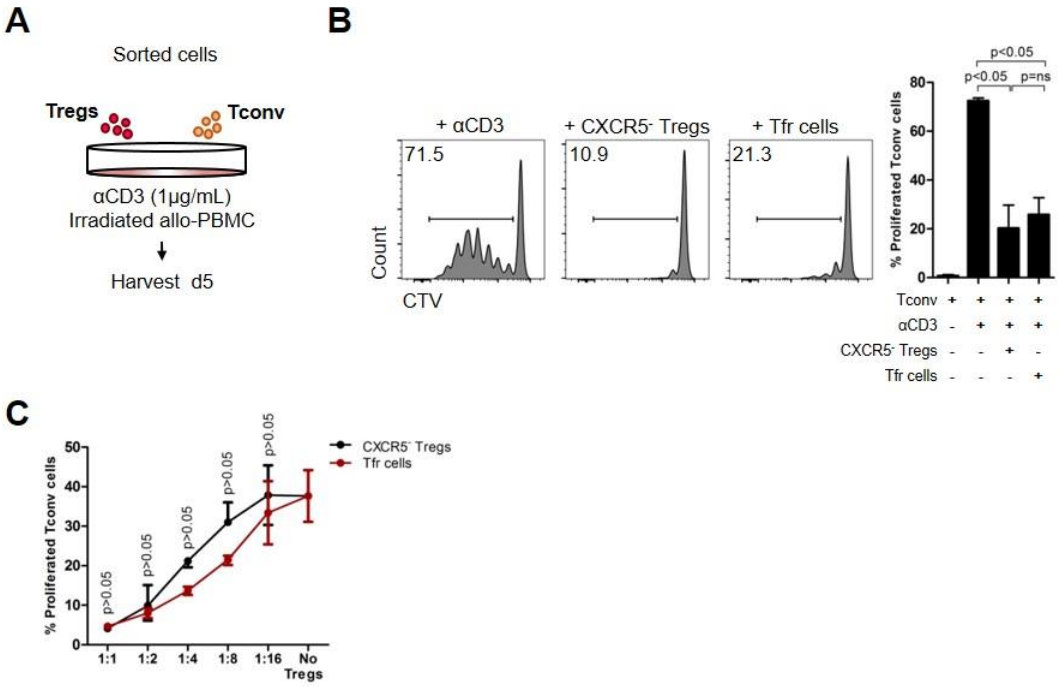


Figure 10: Blood Tfr cells are a distinct subset of suppressive Treg cells.
(A) Schematic representation of *in vitro* suppression assay. FACS-sorted 25 x 10^3 CXCR5^{-}CD25^{-}CD127^{+}CD4^{+} Tconv cells were co-cultured with 25 x 10^3 CXCR5^{-}CD25^{+}CD127^{-}CD4^{+} Tregs or CXCR5^{+}CD25^{+}CD127^{-}CD4^{+} Tfr cells under stimulation by anti-CD3 (1 µg/mL), in presence of 10^5 irradiated (2500 rad) allo-PBMC. After 5 days cells responder cells were analysed for CTV dilution by flow cytometry. **(B)** Proliferation of Tconv cells without Tregs or in the presence of either CXCR5^{-} Tregs or Tfr cells. Representative plots (left) and pooled data (right) (n = 3, each with technical triplicates, One-way ANOVA with post-test Turkey's Multiple Comparison). **(C)** Suppression curve of CXCR5^{-} Tregs and Tfr cells in different ratios, using the same conditions described in Fig. 3A, B) (n = 1, with technical triplicates, Two-way ANOVA). Error bars represent SEM.

Stability of Foxp3 expression is required for the suppressive function of Tregs cells[171]. In order to determine whether blood Tfr cells have stable Foxp3 expression, we stimulated sorted Tfr cells and CXCR5^{-} Treg cells with anti-CD3/CD28 microbeads for 5 days, in the absence of exogenous IL-2. In the absence of IL-2 Treg cells do not survive well in culture. Under these conditions, both CXCR5^{-} Tregs and Tfr cells retain a similar frequency of Foxp3^{+} cells, albeit lower than in the beginning of the culture (**Figure 11A**). Interestingly, the frequency of recovered live Foxp3-expressing cells was slightly higher for sorted Tfr cells as compared to CXCR5^{-} conventional Treg cells. Next, we analysed the relative expression of Foxp3 and CXCR5 in sorted conventional T cells, Tfh cells, Tfr and CXCR5^{-}

Treg cells from human blood, by real-time PCR. Although Foxp3 protein expression was lower in Tfr cells than in CXCR5$^-$ Treg cells (**Figure 6A, B**), Foxp3 gene expression was similar between the two subsets (**Figure 11B**). In addition, circulating Tfh cell and Tfr cells also showed comparable CXCR5 gene expression (**Figure 11B**).

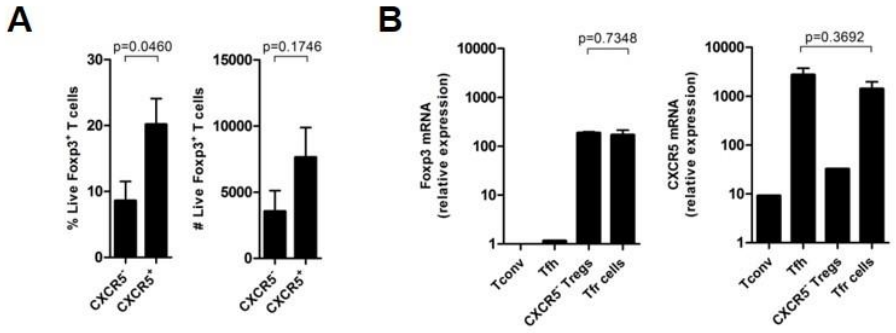


Figure 11: Stability of blood Tfr cells.
(A) Stability of Foxp3 expression by sorted CXCR5$^-$ Tregs and CXCR5$^+$ Tfr cells after 5 days of *in vitro* culture under αCD3/αCD28 (1 µL/well) stimulation. Percentage (left) and cell number (right) (n = 5, each with technical triplicates, Student t-test). **(B)** Relative expression of Foxp3 and CXCR5 by sorted Tconv, Tfh cells, CXCR5$^-$ Tregs and Tfr cells from blood, by real-time RT-PCR. Gene expression normalized to housekeeping genes (B2M, G6PD and ACTB) (n = 2, each with technical duplicates, Student t-test). Error bars indicate SEM.

To investigate whether activation of blood Tfr cells triggers upregulation of Foxp3, CD25 and CTLA-4, a phenomena known to be associated with increased Treg cell suppressive function[171], we analysed the phenotype of sorted CXCR5$^+$ and CXCR5$^-$ Treg cells after 5 days of culture in presence of αCD3/CD28 microbeads. We found an upregulation of Foxp3 and CD25 by Tfr cells, while CTLA-4 was increased in both populations (**Figure 12A**). The levels of expression of these markers by blood Tfr cells after activation resembled those from tissue Tfr cells (compare with **Figure 7B**). Importantly, CXCR5 upregulation was not detected in sorted CXCR5$^-$ Treg cells showing that CXCR5$^+$ Tfr cells are a distinct subset of human blood Treg cells.

Blood Tfr cells do not preferentially suppress humoral responses

In order to address the function of blood Tfr cells we first investigated if this population could directly suppress Tfh cells. Using a similar *in vitro* assay used to prove the regulatory capacity of blood Tfr cells (**Figure 10A**), but with sorted Tfh cells as responders, we found

that blood Tfr cells strongly suppressed Tfh cell proliferation, however without a specific advantage when compared with CXCR5$^-$ Treg cells (**Figure 13A**).

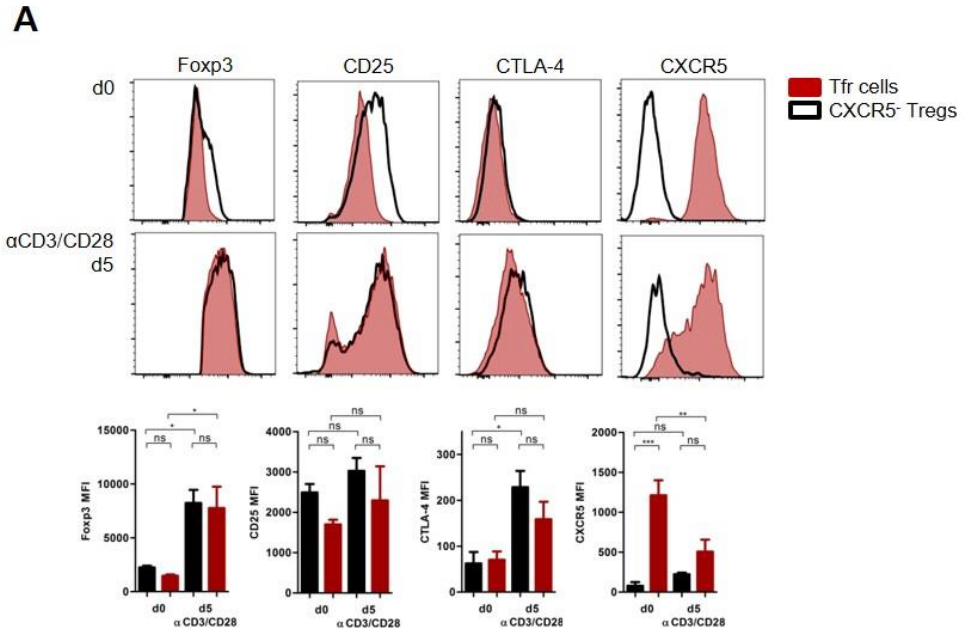


Figure 12: CXCR5 upregulation by blood Treg cells is not induced by TCR or CD28 signalling.
(A) Expression of Foxp3, CD25, CTLA-4 and CXCR5 by sorted CXCR5$^-$ Tregs and Tfr cells at baseline (d0) and after 5 days of *in vitro* culture under αCD3/αCD28 (1 µL/well) stimulation. Representative histograms (top rows) and pooled data (bottom row), from 3 independent experiments, each one with technical triplicates. Pooled data are based on MFI of Foxp3, CD25, CTLA-4 and CXCR5 of sorted CXCR5$^-$ Tregs and Tfr cells at baseline (d0) and after 5 days of *in vitro* culture under αCD3/αCD28 (1 µL/well) stimulation (*p<0.05, **p<0.01, ***p<0.001, n = 3, one-way ANOVA with post-test Turkey's multiple comparison). Error bars indicate SEM. (ns = not significant).

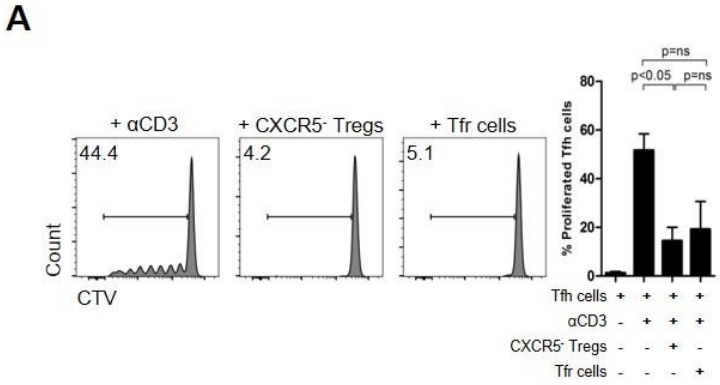


Figure 13: Blood Tfr cells suppress Tfh cell proliferation.
(A) Proliferation of CXCR5$^+$CD25$^-$CD127$^+$CD4$^+$ Tfh cells without regulatory T cells or in the presence of either CXCR5$^-$ Tregs or Tfr cells after 5 days of *in vitro* culture as described in Figure 10A. Representative plots (left) and pooled data (right) (n = 3, each with technical triplicates, One-way ANOVA with post-test Turkey's Multiple Comparison). Error bars indicate SEM. (ns = not significant).

Next, to directly assess the impact of blood Tfr cells on B cell activation, we used *in vitro* T-B co-cultures in presence of SEB superantigen (**Figure 14A**). After 5 days of culture, B cells upregulated CD38 and downregulated IgD only in presence of Tfh cells (**Figure 14B**). Both $CXCR5^-$ and $CXCR5^+$ Treg cells impaired the generation of $CD38^+IgD^-$ GC-like B cells. Consistent with our results from suppression assays with Tfh cells (**Figure 13A**), Tfh cell proliferation was inhibited by $CXCR5^-$ and $CXCR5^+$ Treg cells (**Figure 14B)**. However, in these conditions, blood Tfr cells were less potent compared to conventional $CXCR5^-$ Treg cells in suppression Tfh cell proliferation (**Figure 14C**). As expected Tfh cells showed better proliferation responses in co-culture with B cells.

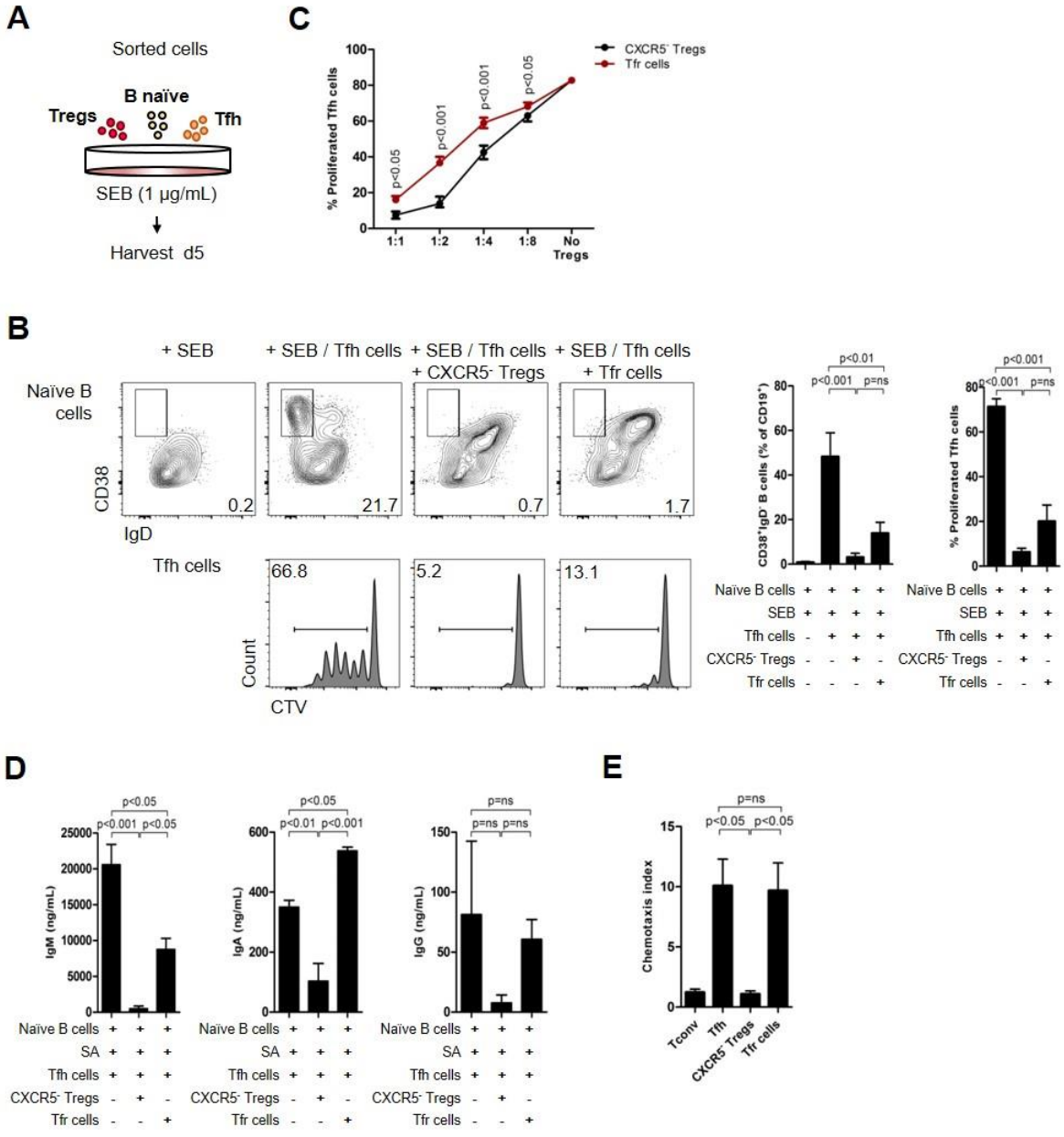


Figure 14 (previous page): Blood Tfr cells do not show specialized humoral regulatory capacity.
(A) Schematic representation of suppression co-culture assay. FACS-sorted 25 x 10^3 $CXCR5^+CD25^-CD127^+CD4^+$ Tfh cells (or $CXCR5^-CD25^-CD127^+CD4^+$ Tconv cells) were co-cultured for 5 days with 25 x 10^3 $CXCR5^+CD25^+CD127^-CD4^+$ Tregs (or $CXCR5^-CD25^+CD127^-CD4^+$ Tregs) under stimulation by SEB (1 µg/mL) and in the presence of 30 x 10^3 $CD27^-IgD^+CD19^+$ naïve B cells. **(B)** Upregulation of CD38 and downregulation of IgD by naïve B cells (top) and proliferation of Tfh cells by CTV dilution (bottom) without Tregs or in the presence of either $CXCR5^-$ Tregs or Tfr cells. Representative plots (left) and pooled data (right) (n = 5, each with technical triplicates, One-way ANOVA with post-test Turkey's Multiple Comparison). **(C)** Suppression curve of $CXCR5^-$ Tregs and Tfr cells in different ratios, using the same conditions described in Fig. 4B, C) (n = 1, with technical triplicates, Two-way ANOVA). **(D)** ELISA determination of for IgA, IgM and total IgG in supernatants after 10 days of *in vitro* co-culture performed as described in (A), but using SEB (1µg/mL) + SEA (10g/mL) + SEE (10ng/mL) + TSST-1 (10ng/mL) as superantigen stimulation. (n = 3, each with technical triplicates, One-way ANOVA with post-test Turkey's Multiple Comparison). **(E)** *In vitro* migration of 75 x 10^3 sorted Tconv, Tfh, $CXCR5^-$ Tregs, and Tfr cells towards a CXCL13 gradient (2 µ/mL), expressed by chemotaxis index (n = 3, each with technical triplicates, One-way ANOVA with post-test Turkey's Multiple Comparison). Error bars indicate SEM. (ns = not significant).

To further address the function of blood Tfr cells on humoral responses we analysed class switch recombination by naïve B cells 10 days after superantigen stimulation. We found that blood Tfr cells, although able to reduce activation of naïve B cells and proliferation of Tfh cells as shown before, did not significantly limit class switch recombination by B cells, as no impact on IgA nor IgG production was observed (**Figure 14D**). On the contrary, $CXCR5^-$ Treg cells efficiently suppressed humoral responses (**Figure 14D**).

CXCR5/CXCL13-dependent migration to GC is critical for suppression of humoral responses by Tfr cells[256,254] and plasma CXCL13 levels have been correlated to ongoing GC responses in humans[447]. To prove that blood Tfr cells were capable to migrate towards a CXCL13 gradient we conducted *in vitro* chemotaxis assays with sorted populations from human peripheral blood. We found that, although the CXCR5 MFI of peripheral Tfh and Tfr cells was slightly different (**Figure 6A, B**), both populations shared their ability to migrate towards a CXCL13 gradient, showing functional capacity of blood Tfr cells to enter CXCL13 enriched tissues (**Figure 14E**).

Blood Tfr cells have a distinctive naïve-like phenotype

To explain the surprising observation that blood Tfr cells do not suppress antibody production we hypothesized that this population could represent thymus-derived precursors of Tfr cells not yet fully committed to regulate humoral responses. Indeed, we found that

blood Tfr cells were predominantly CD45RO$^-$Foxp3^{lo} resting Treg cells (**Figure 15A**), expressing high levels of CD45RA, CCR7, CD62L, CD27 and low levels of HLA-DR, reminiscent of a naïve phenotype (**Figure 15B).**

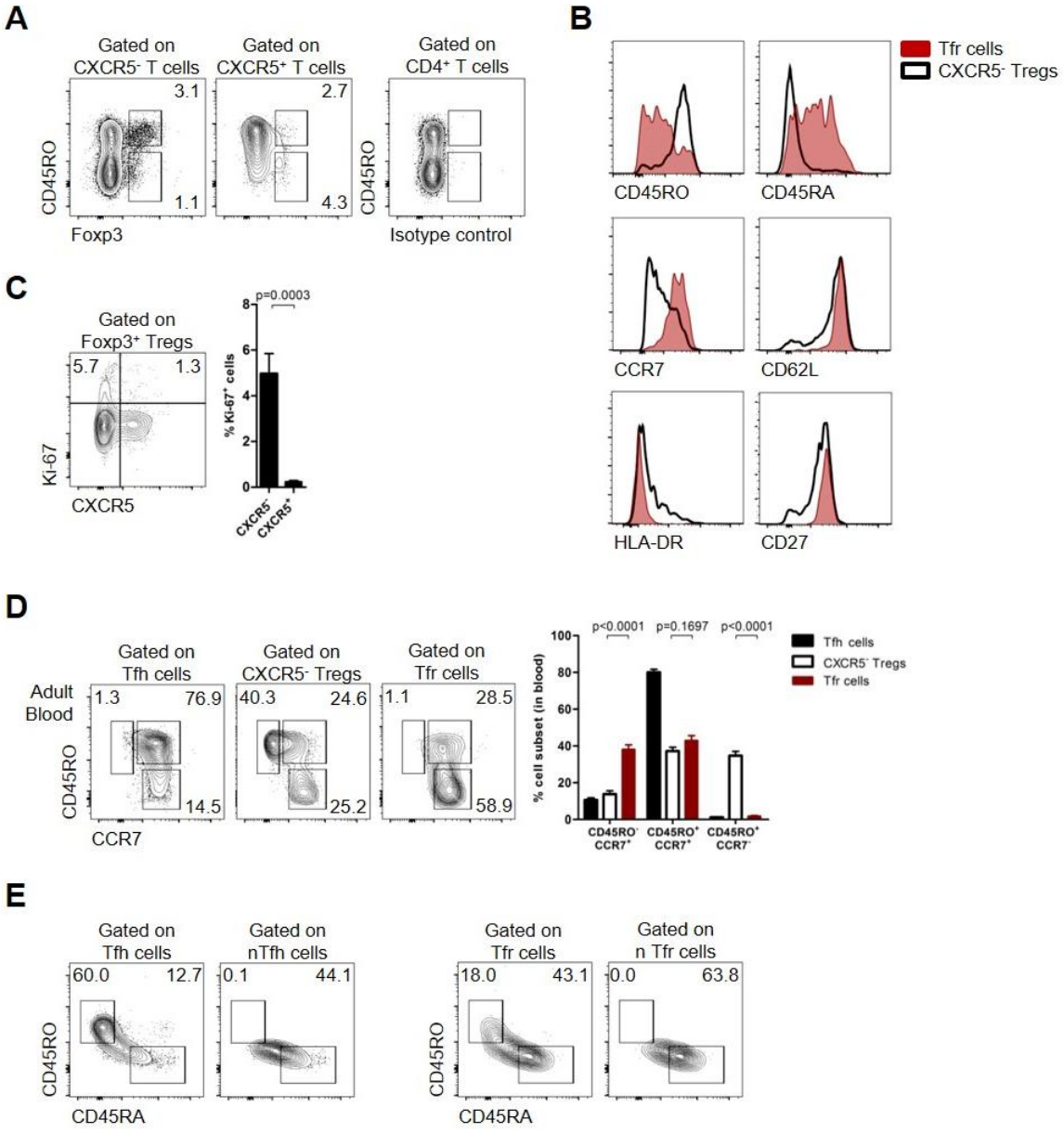


Figure 15: Blood Tfr cells are immature (resting-like) cells.
(A) Backgate of CXCR5$^-$ and CXCR5$^+$ Tregs accordingly to CD45RO and Foxp3 expression. **(B)** Expression of CD45RO, CD45RA, CCR7, CD62L, HLA-DR and CD27 by Tfr cells (red) and CXCR5$^-$ Tregs (black) in blood. **(c)** Expression of Ki-67 by CXCR5$^+$ Tregs and CXCR5$^-$ Tregs in blood (n = 22, Student t-test). **(D)** CD45RO$^+$CCR7$^-$ effector-memory (EM), CD45RO$^+$CCR7$^+$ central-memory (CM) and CD45RO$^-$CCR7$^+$ naïve subsets of Tfr cells and CXCR5$^-$ Tregs in adult blood. Representative plots (left) and pooled data (right) (n = 22, Student t-test). Tfh cells are represented in blue, CXCR5$^-$ Tregs in black and Tfr cells in red. **(E)** Expression of CD45RO and CD45RA in adult blood by total Tfh cells and Tfr cells (left) and by CCR7$^+$CD45RO$^-$ naïve Tfh cells (nTfh cells) and by CCR7$^+$CD45RO$^-$ naïve Tfr cells (nTfr cells) (right). Representative plots from 22 healthy donors. Bars represent SEM. (ns = not significant).

Virtually, all blood Tfr cells were quiescent Ki-67^{-} non-proliferating cells when analyzed *ex vivo* (**Figure 15C**). Moreover, circulating Tfr cells were virtually devoid of CD45RO^{+}CCR7^{-} effector-memory cells in striking contrast to CXCR5^{-} Treg cells, a phenotype more similar to circulating Tfh cells (**Figure 15D**). While the vast majority of blood Tfh cells were CD45RO^{+}CCR7^{+} central-memory cells, consistently with previous reports[52,54–56], a significant proportion of Tfr cells were CD45RO^{-}CCR7^{+} naïve cells (**Figure 15D**). Furthermore, the few CD45RO^{-} Tfh cells did not express high levels of CD45RA indicating that those cells were not really naïve, in contrast to Tfr cells. (**Figure 15E**). Therefore, blood Tfr cells constitute a pool of naïve resting cells.

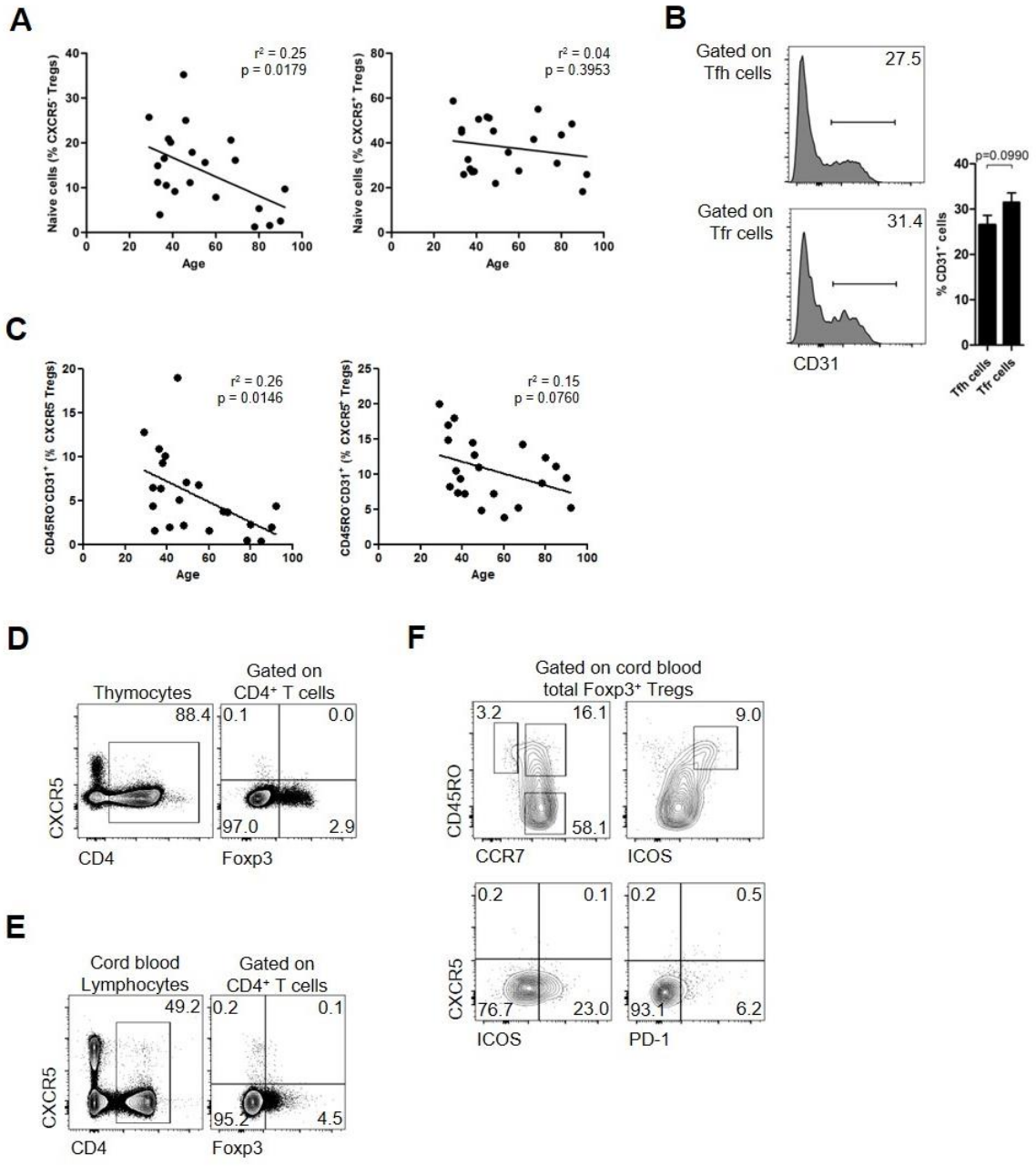


Figure 16 (previous page): Blood Tfr cells are generated in the periphery after mature adaptive immune responses.
(A) Variation of $CD45RO^{-}CCR7^{+}$ naïve Tfr and $CXCR5^{-}$ Treg cells frequency in blood accordingly to age (n = 22, linear regression). **(B)** Variation of $CD45RO^{-}CD31^{+}$ naïve Tfr cells and $CXCR5^{-}$ Treg cells frequency in blood accordingly to age (n = 22, linear regression). **(C)** Expression of CD31 by Tfh and Tfr cells in adult blood of healthy donors. Representative plots (left) and pooled data (right) (n = 22, Student t-test). **(D)** Expression of CXCR5 by $Foxp3^{+}CD4^{+}$ thymocytes (n = 4). **(E)** Expression of CXCR5 by cord blood $Foxp3^{+}CD4^{+}$ T cells (n = 3). **(F)** Expression of CD45RO, CCR7, CXCR5, PD-1, and ICOS by cord blood $Foxp3^{+}$ Treg cells (n = 3). Bars represent SEM.

To test whether blood Tfr cells were indeed thymus-derived precursors of tissue Tfr cells we analysed the frequency of these cells accordingly to age. Contrary to thymic derived naïve Tregs, $CD45RO^{-}CCR7^{+}$ naïve Tfr cells did not significantly decrease with increasing age (**Figure 16A**). In addition, the expression of CD31, a marker used to identify recent thymic emigrants in human blood[245,247,448], was not specifically enriched in that population (**Figure 16B, C**). Although these observations suggest blood Tfr cells are not a thymic population, this was not conclusive. Therefore, we directly examined CXCR5-expressing T cells in the human thymus and neonatal cord blood. There was not a population of $CXCR5^{+}$ Treg cells detected in any of those tissues (**Figure 16D-F**). Although, CXCR5-expressing Tregs were not found in cord blood, some $Foxp3^{+}$ cells expressed CD45RO, suggesting that additional activation signals not present before birth are required to shape a CXCR5 phenotype in circulating Treg cells. Consistent with our previous data, $ICOS^{+}$ Tregs were detected in cord blood, indicating that ICOS cannot be used as a specific follicular marker in circulating human Treg cells (**Figure 16F**).

Blood Tfr cells emerge from lymphoid organs before B-cell interaction

Having demonstrated that circulating Tfr cells did not egress from the thymus, we investigated whether Tfr cells recirculate from secondary lymphoid tissues before being fully committed to tissue Tfr cells. We compared both $CXCR5^{+}$ and $CXCR5^{-}$ Treg cell subsets from children paired blood and tissue (tonsils) concerning their effector-memory, central-memory and naïve composition. We found that $CD45RO^{+}CCR7^{-}$ effector Tfr cells were present in lymphoid tissues but not in blood, suggesting that effector Tfr cells are selectively retained in tissues, similarly to effector Tfh cells (**Figure 17A**). Therefore, it is unlikely that blood $CD45RO^{-}$ Tfr cells derive from the fully mature tissue Tfr cells that express CD45RO,

as the few CD45RA-re-expressing end-stage memory $CD4^+$ T cells do not become $CD45RO^-$ (**Figure 15E**)[449,450].

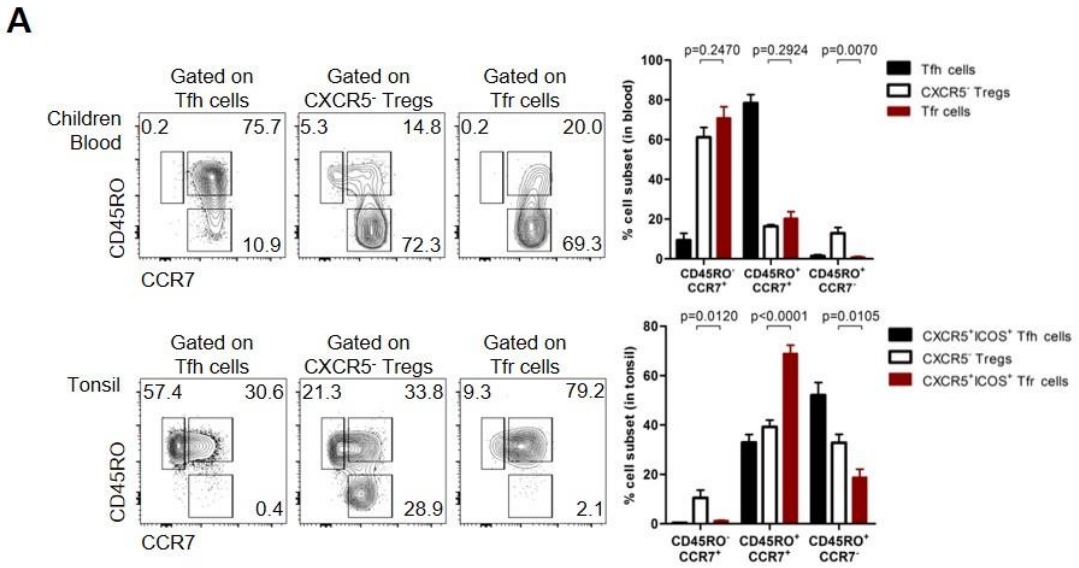


Figure 17: Tissue Tfr cells are effector cells.
(A) $CD45RO^+CCR7^-$ EM, $CD45RO^+CCR7^+$ CM and $CD45RO^-CCR7^+$ naïve subsets of Tfr cells and $CXCR5^-$ Tregs in children blood (top) and in tissues (bottom). Representative plots (left) and pooled data (right) (n = 6, Student t-test). Tfh cells are represented in blue, $CXCR5^-$ Tregs in black and Tfr cells in red. $CXCR5^+$ subsets in tonsils were defined as $CXCR5^+ICOS^+$ cells (Figure 7A). Bars represent SEM.

To directly assess the functional differences between tissue and blood Tfr cells we performed functional assays with sorted Tfr cells from tonsils. While it was possible to sort blood Tfr populations with high purity (**Figure 9B**), similar purities cannot be achieved from tonsils, with the Tfr cells representing a minority of the sorted cells (**Figure 18A, B**). In fact, many Foxp3-negative T cells from the tonsil expressed high levels of CD25 and low CD127. Nevertheless, we performed suppressive assays with those Treg cells sorted from tonsils. We found that tonsil Tfr cells (together with a large number of contaminants, as $Foxp3^+$ Tfr cells comprised only 10% of the total sorted cells) had a suppressive potency similar to conventional Treg cells, which had fewer contaminants (**Figure 18C**). Although, we cannot formally exclude a role of Foxp3-negative contaminant cells for the suppression attributed to tonsil Tfr cells, it is clear that, overall, the tonsil Tfr population had at least comparable suppressive function as Treg cells.

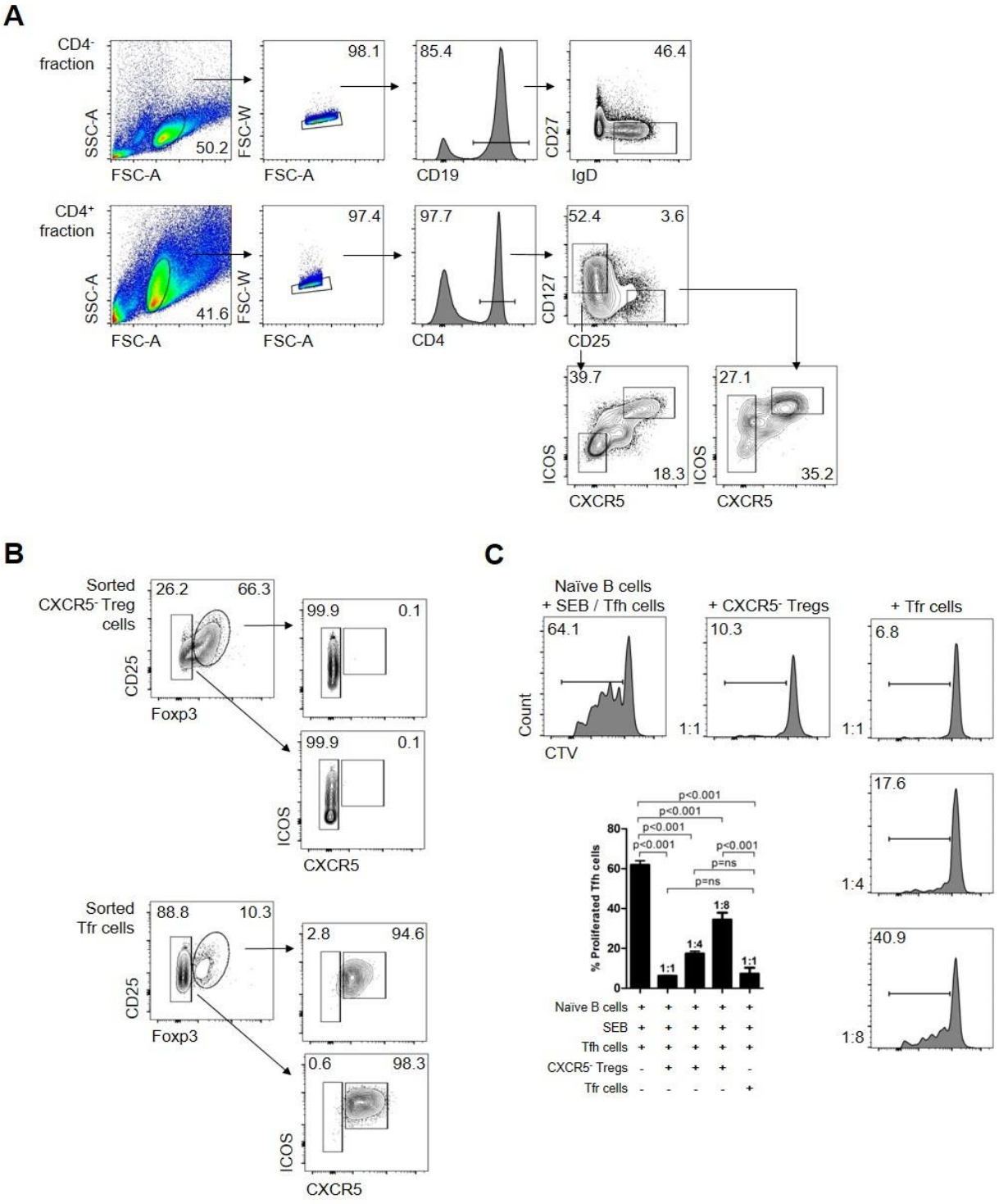


Figure 18: Sorting strategy for human tonsil Tconv, Tfh, CXCR5$^-$ Treg and Tfr cells.
(A) Sorting strategy for FACS-sort of CXCR5$^+$ICOS$^+$CD25$^-$CD127$^+$CD4$^+$ Tfh cell, CXCR5$^-$ICOS$^-$CD25$^-$CD127$^+$CD4$^+$ Tconv cell, CXCR5$^+$ICOS$^+$CD25$^+$CD127$^-$CD4$^+$ Tfr cells, CXCR5$^-$ICOS$^-$CD25$^+$CD127$^-$CD4$^+$ Treg cells from human tonsils. CD27$^-$IgD$^+$CD19$^+$ naïve B cell populations from human tonsils were sorted as described in Figure 9A. **(B)** Purity of FACS-sorted CXCR5$^+$ICOS$^+$CD25$^+$CD127$^-$CD4$^+$ Tfr cells, CXCR5$^-$ICOS$^-$CD25$^+$CD127$^-$CD4$^+$ Treg cells. **(C)** Proliferation of Tfh cells by CTV dilution (bottom) without Tregs or in the presence of either CXCR5$^-$ or Tfr cells (at different ratios), using the same conditions described in Figure 14A. Representative plots (top and right) and pooled data (centre) from one experiment with technical

triplicates, One-way ANOVA with post-test Turkey's Multiple Comparison). (ns = not significant).

Our data suggest that blood Tfr cells are generated in secondary lymphoid tissue prior to full differentiation towards mature Tfr cells. It has been known that full differentiation of follicular T cells requires a two-step process with an initial activation mediated by DCs and a subsequent B cell interaction in the B-T border. We investigated whether blood Tfr cells, given their immature phenotype could be generated before the B cell interactions required for acquisition of terminal differentiation. To investigate this issue, we analyzed peripheral blood from X-linked Agammaglobulinemia (BTK-deficient) patients, with a complete absence of $CD19^+$ cells. We observed a striking decrease in blood Tfh cells in those patients, in line with previous reports (**Figure 19A**)[119]. However, frequency of blood Tfr cells were not decreased in B-cell deficiency patients (**Figure 19A**).

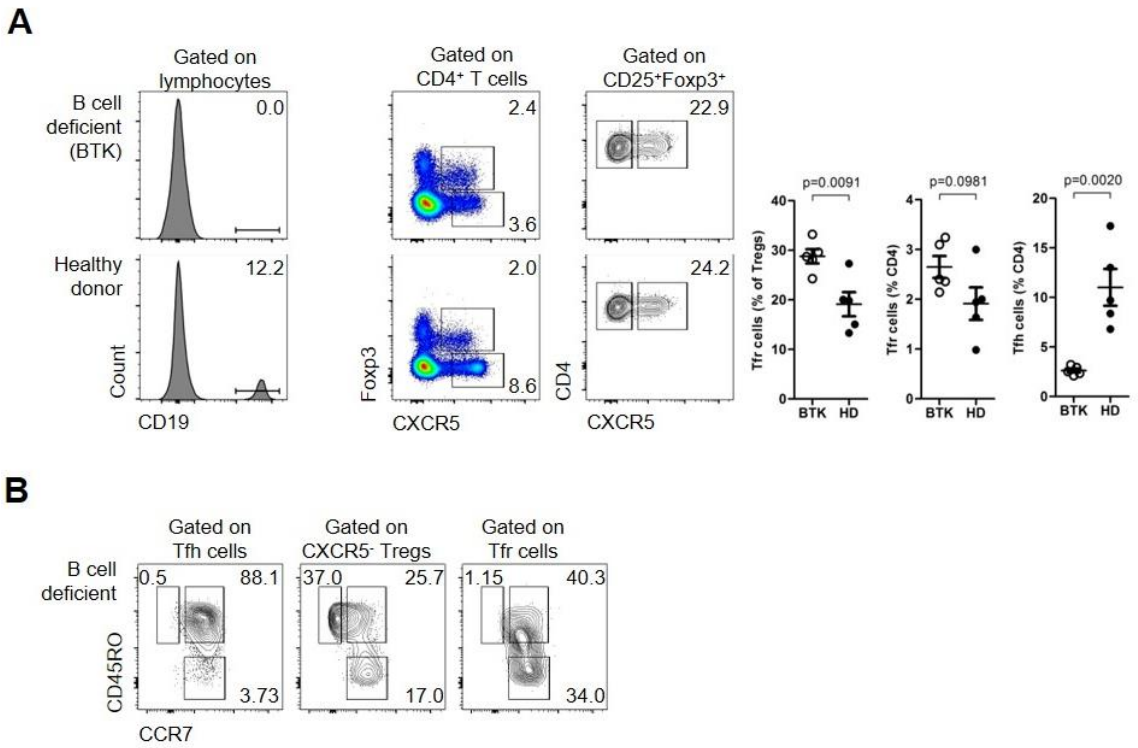


Figure 19: Blood Tfr cells emerge prior to B-cell interactions.
(A) Blood Tfh and Tfr cells from X-linked Agammaglobulinemia (BTK-deficient) patients, compared to sex and age-matched healthy donors. Representative plots (left) and pooled data (right) (n = 5, Student t-test). **(B)** $CD45RO^+CCR7^-$ effector-memory (EM), $CD45RO^+CCR7^+$ central-memory (CM) and $CD45RO^-CCR7^+$ naïve subsets of Tfr cells and $CXCR5^-$ Tregs in peripheral blood of BTK patients. Representative plots of 5 BTK patients. Error bars represent SEM.

These observations are conclusive in establishing that blood Tfr cells enter the circulation before B-cell contact, while most of blood Tfh cells require B cell interactions. To investigate whether CD45RO$^+$ and CD45RO$^-$ blood Tfr cells could discriminate between Tfr cells recirculating before and after B-cell interaction, we analysed these two populations in peripheral blood of patients with B-cell deficiency. No differences were found, suggesting that Tfr cells upregulate CD45RO irrespective of B-cell interaction (**Figure 19B**).

We therefore hypothesized that blood Tfr cells are generated in secondary lymphoid tissue and enter the circulation before full differentiation towards tissue Tfr cells (**Figure 20A**). To test our model *in vivo*, we analysed samples from healthy adults undergoing influenza vaccination. Previous studies have shown a positive correlation between circulating Tfh cell subsets and antibody responses following influenza vaccination in healthy adults[56,120,123]. We, therefore, analysed the impact of vaccination in circulating Tfr cells. Consistent with our hypothesis we found that circulating Tfr cells increased on day 7 following influenza vaccination (**Figure 20B**). This observation is in line with our prediction that during ongoing GC responses Tfr cells are generated and some exit from the tissue to the peripheral blood.

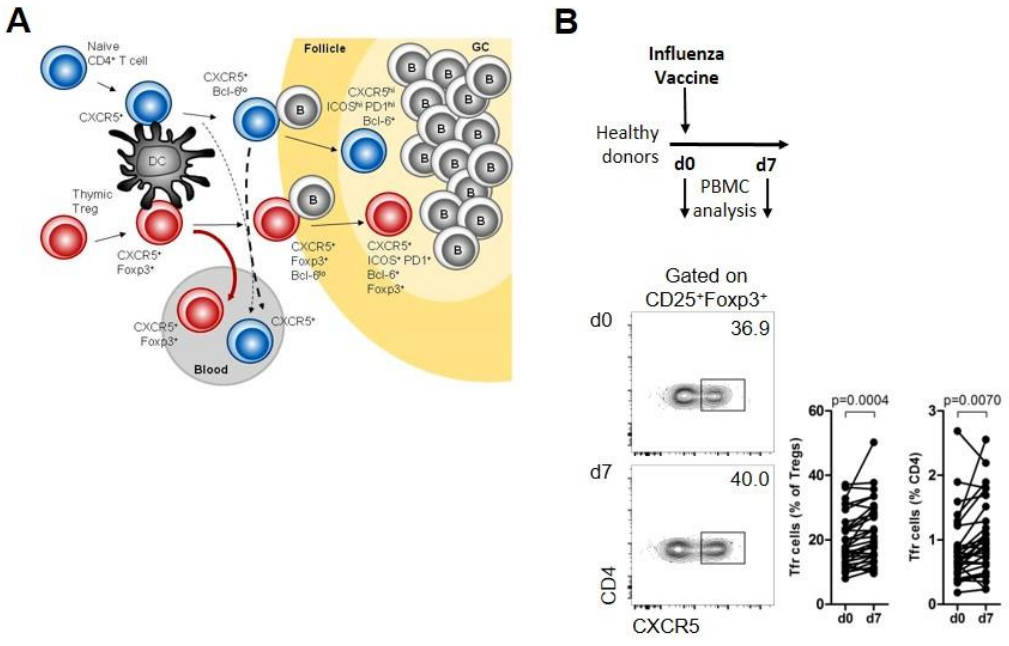


Figure 20: Blood Tfr cells are lymphoid tissue derived Tfr precursors.
(A) Model of CXCR5$^+$ follicular helper and regulatory cells T cells generation and recirculation in humans, upon antigen stimulation. Tfh cells in red and Tfr cells in blue. **(B)** Frequency of peripheral blood Tfr cells on the day of influenza vaccination (d0) and 7 days later in healthy volunteers. Schematic representation and representative plots (left) and pooled data (right) (n = 32, paired Student t-test).

Discussion

Our comprehensive evaluation of human Tfr cells supports a model in which blood Tfr cells are generated following the initial steps that lead to GC responses in secondary lymphoid tissues, exiting the tissue prior to interactions with B cells that are required for complete differentiation towards tissue resident Tfr cells.

Although some studies have quantified blood CXCR5$^+$ Treg cells as circulating Tfr cells in different diseases, the human biology of CXCR5$^+$ Treg cells remains elusive[329,333,334,338,341]. Moreover, most of the literature describe studies where what is defined as blood Tfh cells contain both Tfh and CXCR5$^+$ Tfr cells, while many other studies identify as Treg cells a mixture of *bona fide* conventional Treg cells together with CXCR5$^+$ Tfr cells. As such, results may be confounded by combining effector and regulatory cell populations.

We found that Tfr cells in tonsils have follicular and regulatory markers and were found within GC, whereas blood Tfr cells do not express ICOS, PD-1, or Bcl-6, apparently diverging these cells from follicular imprinting. Previous studies have described low ICOS and PD-1 expression, and no Bcl-6 expression in human blood Tfh cells [52]. In mice, blood Tfr cells have also lower expression of ICOS [258]. It was also reported that murine circulating Tfr cells can bypass the B cell zone and do not gain full activation as part of a memory programmed state[258]. In line with these studies, the absence of ICOS, PD-1 and Bcl-6 from human blood CXCR5$^+$ Tfr cells does not exclude their follicular ontogeny.

Our results show key differences between mice and humans regarding the function of blood CXCR5$^+$ Tfr cells: while murine blood Tfr cells appear to be specialized in suppressing antibody production (despite their lower suppressive capacity when compared to tissue Tfr cells)[258–260,272], human blood Tfr cells do not have the ability to fully suppress humoral responses. Nevertheless, we found that blood Tfr cells specifically migrated towards CXCL13 gradient, suggesting these cells have the capacity to reach the follicles. Importantly, CXCR5$^-$ conventional Tregs did not upregulate CXCR5 upon *in vitro* activation, further confirming CXCR5-expressing Tfr cells as a distinctive Treg cell subset. Moreover, our results from vaccination cohorts show that blood Tfr cells are indicative of ongoing humoral activity.

We also found that blood Tfr cells have a prominent naïve phenotype. However, they are absent from the thymus and cord blood (where activated Treg cells can already be found). These observations provide compelling evidence that activation signals generated in peripheral lymphoid organs are required to shape a $CXCR5^+$ phenotype on human $Foxp3^+$ T cells. Conversely, tissue Tfr cells are almost all $CD45RO^+$ antigen experienced effector cells. Taken together, these observations led us to hypothesize that blood Tfr cells leave lymphoid tissues as immature cells, prior to B cell interaction in T-B border, and full differentiation into Tfr cells. This view was supported by the presence of blood Tfr cells in peripheral blood of patients lacking B-cells due to genetic defects. This finding provides an explanation for the incomplete suppressive function of blood Tfr cells.

Given that circulating Tfr cells have an immature phenotype, it is not surprising that blood Tfr cells are not fully endowed with suppressive function, because the suppressive capacity of conventional Treg cells have been ascribed predominantly to those cells with a more mature phenotype. The TCR repertoire of Tfr cells is different from Tfh and probably skewed towards auto-antigens[264], it is possible that circulating Tfr cells represent a pool of cells ready to be recruited into subsequent GC responses as they retain the ability to migrate towards CXCL13. An important limitation of our study is the difficulty to isolate tissue Tfr cells for functional assays, as CD25 and CD127 are not reliable to identify tonsil Foxp3-expressing cells. Therefore, we were not able to directly address whether more mature Tfr cells sorted from GC show a specialized humoral suppressive capacity in humans.

In conclusion, our data support a model in which $CXCR5^+Bcl6^-$ T cells egress from secondary lymphoid tissues during antigen-driven immune responses. While, the frequency of blood Tfh cells is reduced in the absence of B cells, Tfr cells do not require interactions with B cells. Thus, the acquisition of a $CXCR5^+Foxp3^+$ phenotype in the tissues precedes access to the follicle, where the cells acquire a fully mature phenotype. Consequently, circulating Tfr cells represent lymphoid tissue derived Tfr precursors not yet endowed with full B-cell and humoral regulatory function.

Blood Tfr/Tfh Ratio Marks Ectopic Lymphoid Structure Formation in Primary Sjögren Syndrome

Introduction

Sjögren's syndrome is characterized by lymphocytic infiltration of salivary and lachrymal glands, leading to xerostomia and keratoconjunctivitis sicca[437,439,451]. However, like in other autoimmune diseases, disease heterogeneity remains a hurdle to the development of better therapeutic approaches. Disease manifestations are mediated by complex mechanisms involving innate type I IFN signature and autoantibodies produced during dysregulated adaptive immune responses[451]. ELS[143,356,357] found in salivary and lachrymal glands are a major site of autoantibody production[415,420,451]. These B – T lymphocyte aggregates define focal sialadenitis (FSA), the histological hallmark of SS, and have prognostic significance[419,430]. Nevertheless, many patients fulfil classification criteria for SS with almost normal salivary gland histology, which highlights the clinical heterogeneity of this disease. Whether clinical heterogeneity could be explained by different immunopathogenic mechanisms is unknown. However, it is reasonable to hypothesize that patients with FSA, especially those with ectopic GC formation, constitute a subgroup of patients more suitable for therapies targeting T – B interactions.

Dysregulated GC reactions in secondary and tertiary lymphoid organs underlie the generation of self-reactive autoantibodies and many aspects of autoimmune diseases[143,357]. GC reactions are orchestrated mainly by T follicular helper (Tfh) and T follicular regulatory (Tfr) cells. Tfh cells provide cognate help to B cells, thus promoting their clonal selection and affinity maturation[63]. Conversely, Tfr cells regulate and limit the GC reaction assuring antigen-specific antibodies are produced[257,256,254,255,331]. While the precise mechanisms of Tfr cell functions are not fully understood, unbalanced Tfh and Tfr cell responses may prompt antibody-mediated autoimmune diseases.

In the previous section, I described that blood Tfr cells are immature and not yet fully specialized in suppressing humoral responses[330]. However, the relationship between blood and tissue Tfr cells remains elusive in patients with immune-mediated diseases. Herein we

investigated the potential role of blood Tfr and Tfh cells as biomarkers of ectopic lymphoid activity in the target organ of SS.

Results

Blood activated PD-1^{+}ICOS^{+} Tfh cells correlate with SS disease activity

To address the impact of Tfr and Tfh cells in human autoimmunity, we studied primary Sjögren syndrome from a cohort of 56 patients referred for MSG biopsy due to clinical suspicion of SS, together with a cohort of age and sex-matched healthy donors (**Figure 4, Table 6, 7**).

Table 6: Summary of demographic characteristics of primary Sjögren syndrome (SS), non-Sjögren sicca syndrome (non-SSS) patients, and healthy donors

	Primary SS n = 16	**Non-SSS** n = 16	**Healthy donors** n = 16	**p-value**
Age, y	52.3 ± 17.7	47.3 ± 14.9	48.3 ± 19.7	ns
Gender (f / m), n	15 / 1	15 / 1	11 / 5	ns
Disease duration, y	4.8 ± 6.5	-	-	N/A
ESSDAI	3.8 ± 5.0	-	-	N/A
Extraglandular involvement, n (%)	12 (75)	-	-	N/A
Prednisolone (≤5mg/d), n (%)	5 (31)	0	-	0.0434
Hydroxychloroquine, %	3 (19)	0	-	ns
ANA positive, n (%)	15 (94)	2 (13)	-	<0.0001
Anti-SSA positive, n (%)	15 (94)	0		<0.0001
Anti-SSA/Ro52 titer, UQ	7824 ± 15430	-	-	N/A
Anti-SSA/Ro60 titer, UQ	8728 ± 6079	-	-	N/A
Anti-SSB positive, n (%)	10 (63)	0		<0.0001
Anti-SSB titer, UQ	1206 ± 4236	-	-	N/A
RF positive, n (%)	8 (50)	1 (6)		0.0024
RF titer, UI/mL	45.7 ± 26.1	18.7 ± 0.0	-	N/A
CRP, mg/dL	0.53 ± 0.58	0.52 ± 0.78	-	ns
ESR, mm	43.7 ± 25.6	22.1 ± 16.3	-	0.010
γ-fraction, g/dL	1.76 ± 0.58	1.07 ± 0.31	-	0.002
MSG histology, n (%)				
Focal sialadenitis	7 (44)	0	-	0.0068
Unspecific inflammation	6 (38)	5 (31)	-	ns
Normal	3 (19)	11 (69)	-	0.0113

Legend: NA, not applicable; ns, not significant

Table 7: Demographic and clinical characteristics of Sjögren syndrome (SS) and non-Sjögren sicca syndrome (non-SSS) patients

Table 7: Demographic and clinical characteristics of primary Sjögren's syndrome (SS) and non-Sjögren sicca syndrome (non-SSS) patients

Patient	Gender, Age	Diagnosis	ESSDAI	ANA	Anti-Ro52	Anti-Ro60	Anti-La	RF	γ-fraction	ESR	CRP	SG Biopsy	Treatment
1	F, 50	pSS	10	1/640	59	13748	17069	16	2.4	85	2.29	LI	-
2	F, 27	pSS	16	1/1280	3392	13748	129	49	1.8	30	0.37	LI	-
3	F, 66	pSS	0	1/320	1024	13748	223	<14	1.5	89	1.18	FSA	-
4	F, 57	pSS	0	1/640	0	0	0	<14	1.1	12	0.04	FSA	-
5	F, 62	pSS	0	0	722	9761	741	<14	1.1	24	0.05	Normal	HCQ
6	F, 33	pSS	0	1/160	478	0	0	<14	1.8	47	0.2	Normal	-
7	F, 67	pSS	1	1/320	44	5666	0	<14	1.4	62	0.86	FSA	-
8	F, 17	pSS	3	1/640	8483	13748	515	30.8	1.9	27	0.34	Normal	-
9*	F, 51	pSS	0	1/160	+	+	+	<14	1.5	NA	NA	LI	PDN
10	F, 49	pSS	7	1/320	5898	13748	184	31.9	2.9	70	0.44	FSA	HCQ, PDN
11	F, 46	pSS	0	1/320	476	3095	0	60.2	1.2	30	0.22	FSA	-
12	F, 78	pSS	5	1/640	3587	13748	374	101.2	NA	30	0.34	FSA	HCQ, PDN
13	M, 40	pSS	2	1/640	1437	13748	34	<14	1.5	22	0.05	LI	-
14	F, 79	pSS	6	1/160	58985	13748	0	44.8	2.1	69	0.76	LI	-
15	F, 69	pSS	11	1/320	27785	0	0	31.6	2.8	47	0.39	LI	PDN
16	F, 45	pSS	0	1/640	12810	11138	29	<14	1.3	12	0.43	FSA	PDN
17	F, 29	Non-SSS	-	0	0	0	0	<14	NA	NA	NA	Normal	-
18	F, 26	Non-SSS	-	0	0	0	0	<14	0.8	9	0.72	Normal	-
19	F, 32	Non-SSS	-	0	0	0	0	<14	0.9	15	0.41	Normal	-
20	F, 72	Non-SSS	-	0	0	0	0	<14	1.5	59	0.13	Normal	-
21	F, 36	Non-SSS	-	0	0	0	0	<14	1.7	26	0.04	Normal	-
22	F, 52	Non-SSS	-	0	0	0	0	NA	1.0	5	0.05	LI	PDN
23	F, 39	Non-SSS	-	0	0	0	0	18.7	NA	18	0.04	LI	-
24	F, 45	Non-SSS	-	0	0	0	0	<14	1.2	25	NA	Normal	-
25	M, 59	Non-SSS	-	0	0	0	0	<14	0.7	3	0.04	Normal	-
26	F, 61	Non-SSS	-	0	0	0	0	<14	0.8	3	0.81	Normal	-
27	F, 48	Non-SSS	-	0	0	0	0	<14	NA	29	0.04	Normal	-
28	F, 30	Non-SSS	-	0	0	0	0	<14	1.1	18	0.06	Normal	-
29	F, 60	Non-SSS	-	0	0	0	0	<14	0.9	16	0.08	LI	-
30	F, 52	Non-SSS	-	1/160	0	0	0	<14	1.2	29	0.27	Normal	-
31	F, 61	Non-SSS	-	1/80	0	0	0	<14	NA	25	2.28	LI	-
32	F, 68	Non-SSS	-	0	0	0	0	<14	NA	52	2.25	LI	-

Legend: CRP, C-reactive protein (mg/dl); ESSDAI, EULAR Sjögren's syndrome disease activity index; ESR, erythrocyte sedimentation rate (mm); F, female; FSA, focal sialadenitis; HCQ, hydroxychloroquine; LI, unspecific lymphocytic infiltration; M, male; NA, not available; non-SSS, Non-Sjögren's sicca syndrome; PDN, prednisolone; pSS, primary Sjögren's syndrome; RF, rheumatoid factor (UI/mL); SG, salivary gland; γ-fraction, serum electrophoresis gamma-fraction (g/dL). * Antibody titers not available.

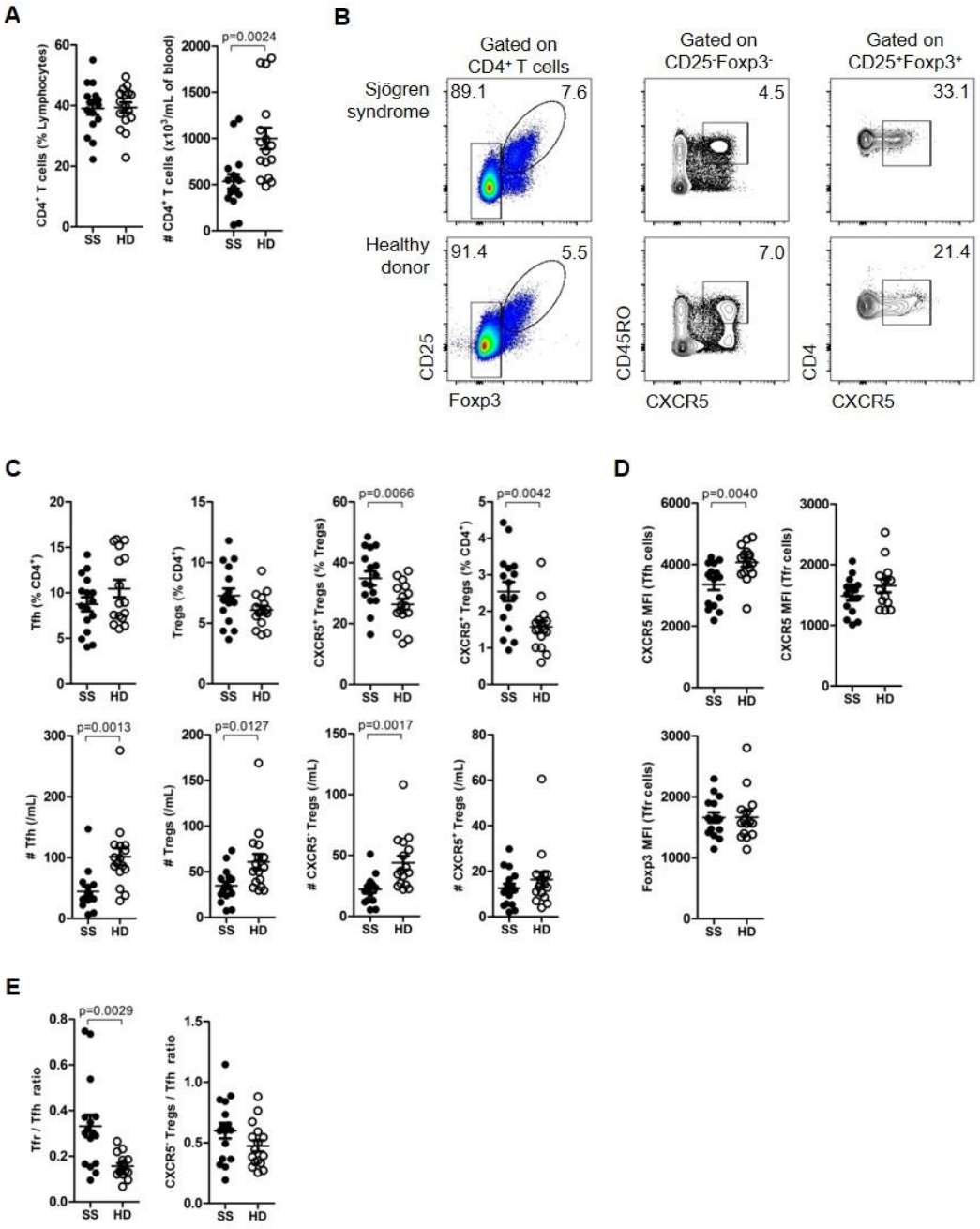


Figure 21: Primary Sjögren's syndrome patients have an increased blood Tfr/Tfh ratio.
(A) Frequency and absolute number (per mL of blood) of $CD4^+$ T cells in peripheral blood of primary S Sjögren's syndrome (SS, n=16) and healthy donors (HD, n=16). **(B)** Identification of $CD25^+Foxp3^+$ Treg, $CXCR5^+CD45RO^+CD25^-Foxp3^-$ Tfh, and $CXCR5^+CD25^+Foxp3^+$ Tfr cells in peripheral blood of SS (n=16) and HD (n=16). Unpaired Student T-test with Welch's correction for variance. Representative plots (left) and pooled data (right). **(C)** Frequency (top) and absolute number (per mL of blood) (bottom) of Tfh, Treg, $CXCR5^-$ Treg, and Tfr cells in peripheral blood of SS patients (n=16) and HD (n=16). Unpaired Student T-test. **(D)** Mean fluorescence intensity of CXCR5 and Foxp3 in peripheral blood Tfh and Tfr cells in SS (n=16) and HD (n=16). Unpaired Student T-test. **(E)** Blood Tfr/Tfh (left) and $CXCR5^-$ Treg/Tfh (right) ratios in SS (n=16) and HD (n =16). Unpaired Student T-test. Bars on scatterplots represent SEM.

Primary SS patients were lymphopenic, a common feature of human systemic autoimmune diseases (**Figure 21A**)[452]. Unexpectedly, we found a striking increased frequency of blood Tfr cells in SS as compared to age-matched healthy donors (**Figure 21B, C)**. Interestingly, while primary SS patients had decreased absolute number of all $CD4^+$ T cell subsets, in line with their $CD4^+$ T cell lymphopenic state, the absolute number of Tfr cells were not different when compared to healthy donors (HD), suggesting that the Tfr cell subset is indeed over-represented in these patients (**Figure 21C**). The increased frequency of the $CXCR5^+$ Treg cell subset was specifically increased providing an explanation for the high Treg cell frequency observed in SS patients (**Figure 21C**). At the protein level, we did not find differences in CXCR5 and Foxp3 expression on Tfr cells from SS patients and HD (**Figure 21D**). While, Tfh cells from SS patients expressed lower CXCR5, we did not test whether Tfh cells from SS patients have different homing properties (**Figure 21D**). Primary SS patients showed a significantly increase in the blood Tfr/Tfh ratio compared to HD (**Figure 21E**). The increase in this ratio was specifically attributed to an increase of Tfr cells, as no differences were found on $CXCR5^-$ Treg cells/Tfh ratio (**Figure 21E**).

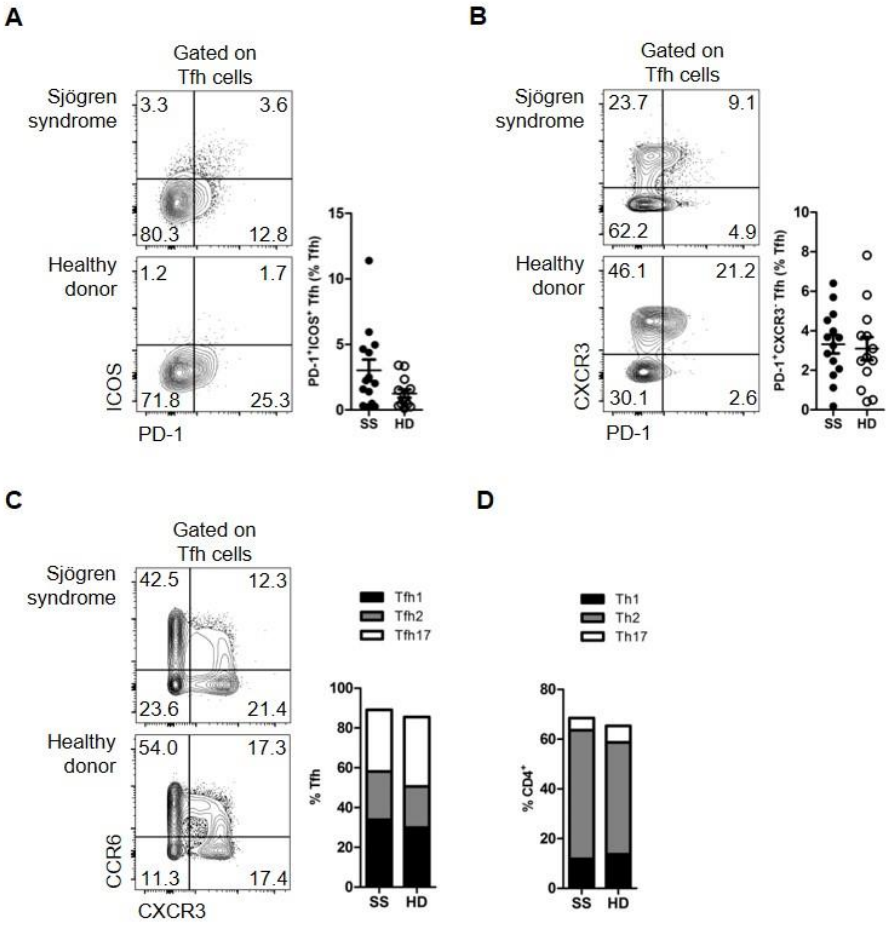


Figure 22 (previous page): Circulating Tfh cell subsets are not altered in primary Sjögren syndrome.
(A) Frequency of PD-1^{+}ICOS^{+} Tfh cells in peripheral blood of SS (n=14) and HD (n=13). Unpaired Student T-test with Welch's correction for variance. Representative plots (left) and pooled data (right). **(B)** Frequency of PD-1^{+}CXCR3^{-} Tfh cells in peripheral blood of SS (n=14?) and HD (n=13). Unpaired Student T-test. Representative plots (left) and pooled data (right). **(C)** Distribution of CCR6^{+}CXCR3^{-} Tfh17-like cells, CCR6^{-}CXCR3^{+} Tfh1-like cells, and CCR6^{-}CXCR3^{-} Tfh2-like cells in peripheral blood of SS (n=16) and HD (n=16). Unpaired Student T-test. **(D)** Distribution of CCR6^{+}CXCR3^{-} Th17 cells, CCR6^{-}CXCR3^{+} Th1 cells, and CCR6^{-}CXCR3^{-} Th2 cells in peripheral blood of SS (n=16) and HD (n=16). Unpaired Student T-test. Bars on scatterplots represent SEM.

We did not find a significant increase of activated PD-1^{+}ICOS^{+} Tfh cells or PD-1^{+}CXCR3^{-} GC-like Tfh cells in SS patients (**Figure 22A, B**)[52,54–56,120]. Contrary to previous reports[453], SS patients had a normal distribution of Tfh1, Tfh2 and Tfh17-like cells (**Figure 22C, D)**.

To investigate whether follicular T cell subsets correlated with autoantibodies, inflammatory markers and disease activity we conducted correlation heatmaps. We found that blood Tfr/Tfh ratio and PD-1^{+}ICOS^{+} Tfh cells significantly correlated with the presence of anti-SSA/Ro60 (r=0.580, p=0.0298) and anti-SSA/Ro52 (r=0.558, p=0.047), respectively (**Figure 23A, B**). While most correlations were barely significant, we found a very strong association between frequency of activated PD-1^{+}ICOS^{+} Tfh cells and disease activity (ESSDAI, r=0.855, p=0.0008) (**Figure 23A, B**). Interestingly, blood Tfr/Tfh ratio and PD-1^{+}ICOS^{+} Tfh cells consistently correlated in opposite directions with the variables analysed, albeit not always reaching statistical significance (**Figure 23A**).

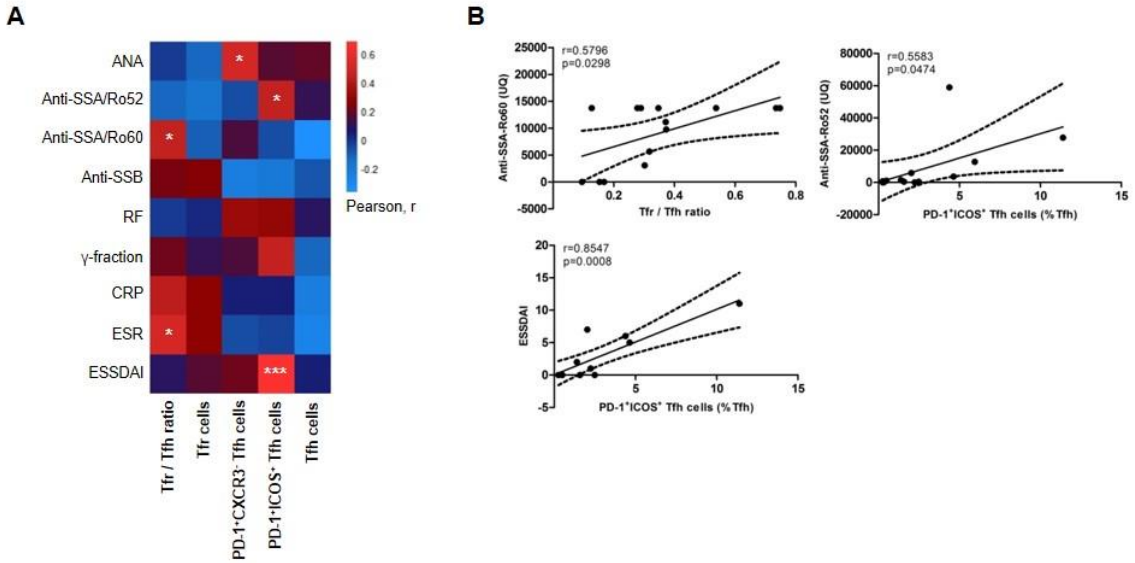


Figure 23 (previous page): Blood PD-1$^+$ICOS$^+$ activated Tfh and Tfr/Tfh ratio marks distinct features of primary Sjögren syndrome.
(A) Heatmap representation of correlation between peripheral blood Tfh cells, PD-1$^+$ICOS$^+$ Tfh cells, PD-1$^+$CXCR3$^-$ Tfh cells, Tfr cells, and Tfr/Tfh ratio and serum autoantibodies titers (antinuclear antibodies (ANA), anti-SSA/Ro52, anti-SSA/Ro60, anti-SSB), rheumatoid factor (RF, IU/mL), serum electrophoresis gamma-fraction (γ-fraction, g/dL), C-reactive protein (CRP, mg/dL), erythrocyte sedimentation rate (ESR, mm/1st hour) and disease activity measured by European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI), in SS patients (n = 16, Pearson coefficient, r). **(B)** Correlation between peripheral blood Tfr/Tfh ratio and serum anti-SSA/Ro60 titers (left); correlation between blood PD-1$^+$ICOS$^+$ Tfh cells and serum anti-SSA/Ro52 (centre) and ESSDAI (right) (n = 16, linear regression with interpolated 95% confidence interval curves). On linear regression plots, dashed line represents interpolated 95% confidence interval.

Blood Tfr/Tfh ratio identifies pathological lymphocytic infiltration in SS target organ

As the Tfr/Tfh ratio may predict the outcome of GC reactions[257,260], we hypothesized that patients with autoimmune diseases with abnormal GC reactions leading to autoantibody production may have an altered Tfr/Tfh ratio. As GC reactions take place in lymphoid tissues and ectopic (or tertiary) lymphoid structures, we studied MSG tissue biopsies from patients with primary SS and controls with non-Sjögren sicca syndrome (**Figure 4, Table 6, 7)**.

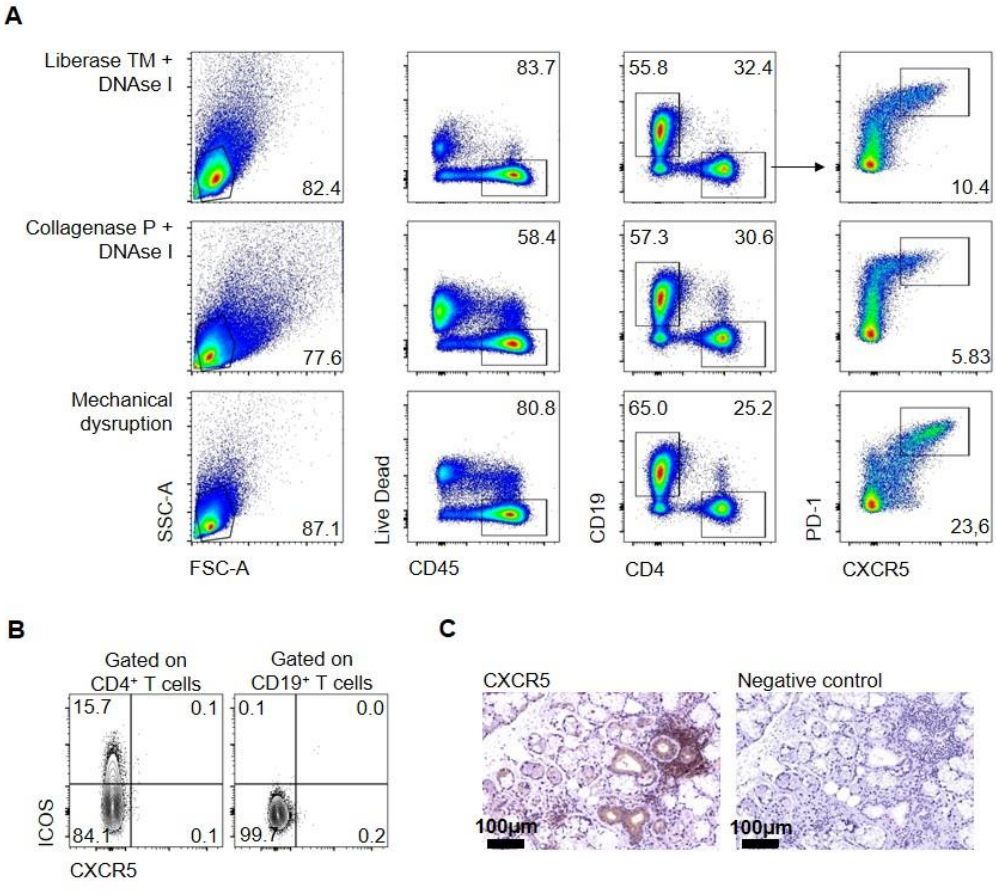


Figure 24 (previous page): Optimization of salivary gland enzymatic digestion.
(A) Impact of different enzymes in membrane markers used for identification of cellular components of minor salivary gland tissue infiltrates, including CXCR5-expressing T cells. Human tonsil fragments from healthy children were used for optimization. Liberase TM (0.1 mg/mL), DNAse I (0.1 mg/mL), Collagenase P (0.1 mg/mL). All enzymes led to significant duction of CXCR5 on PD-1$^+$CD4$^+$ T cells. Representative plots of 2 independent experiments. **(B)** CXCR5 and ICOS expression by salivary gland CD4$^+$ B cells and CD19$^+$ T cells after enzymatic digestion by Liberase TM (0.1 mg/mL) plus DNAse I (0.1 mg/mL), confirming the loss of CXCR5 staining. Representative plots of 14 MSG biopsies. **(C)** Microscopy of formalin-fixed paraffin-embedded MSG stained for CXCR5 (brown, right), with correspondent negative control (left) by immunohistochemistry. Data are representative of MSG sections from 4 SS patients.

Loss of CXCR5 receptor following enzymatic digestion due to a tissue-intrinsic factor (as the same enzymatic digestion did not limit detection of CXCR5 expression on lymphocytes isolated from human tonsils and CXCR5 was detected in salivary gland tissue by immunohistochemistry) excluded direct analysis of tissue Tfh and Tfr cells (**Figure 24A – C)**.

Primary SS patients had an increased frequency and absolute number (per mg of tissue) of non-epithelial CD45$^+$ cells and CD19$^+$ B cells (**Figure 25A – C)**. As around 90% of PD-1$^+$ICOS$^+$ T cells in lymphoid tissues were CXCR5$^+$ Tfh cells, we assessed salivary gland infiltration by PD-1$^+$ICOS$^+$ T cells (**Figure 25D, E**). We found a striking increased frequency and absolute number of infiltrating PD-1$^+$ICOS$^+$ T cells in SS patients (**Figure 25E**). Although, it is likely that this cell population comprise tissue Tfh cells, we cannot exclude the existence of CXCR5$^-$PD-1$^+$ICOS$^+$ T cells, described as a specific type of helper T cells within ELS of RA patients[408]. In both cases, our findings suggest that MSG tissue from primary SS patients host active T-dependent humoral responses.

To validate our results, we compared MSG analysis by flow cytometry with routine histological diagnosis of the same biopsies. Infiltration by CD19$^+$ B cells was found in all SS patients irrespectively of salivary gland morphology. Yet, only patients with ELS/FSA had a significantly higher infiltration by B cells as compared to MSG of patients with non-Sjögren sicca syndrome (**Figure 25F, G**).

When we compared blood Tfh and Tfr populations with lymphocytic MSG infiltration of patients with SS, we found a strong correlation between blood Tfr/Tfh ratio and the presence of ELS in exocrine glands. Indeed, blood Tfr/Tfh ratio was positively correlated with tissue infiltration by CD4$^+$ T cells (r=0.8475, p=0.0039), PD-1$^+$ICOS$^+$ T cells (r=0.8400,

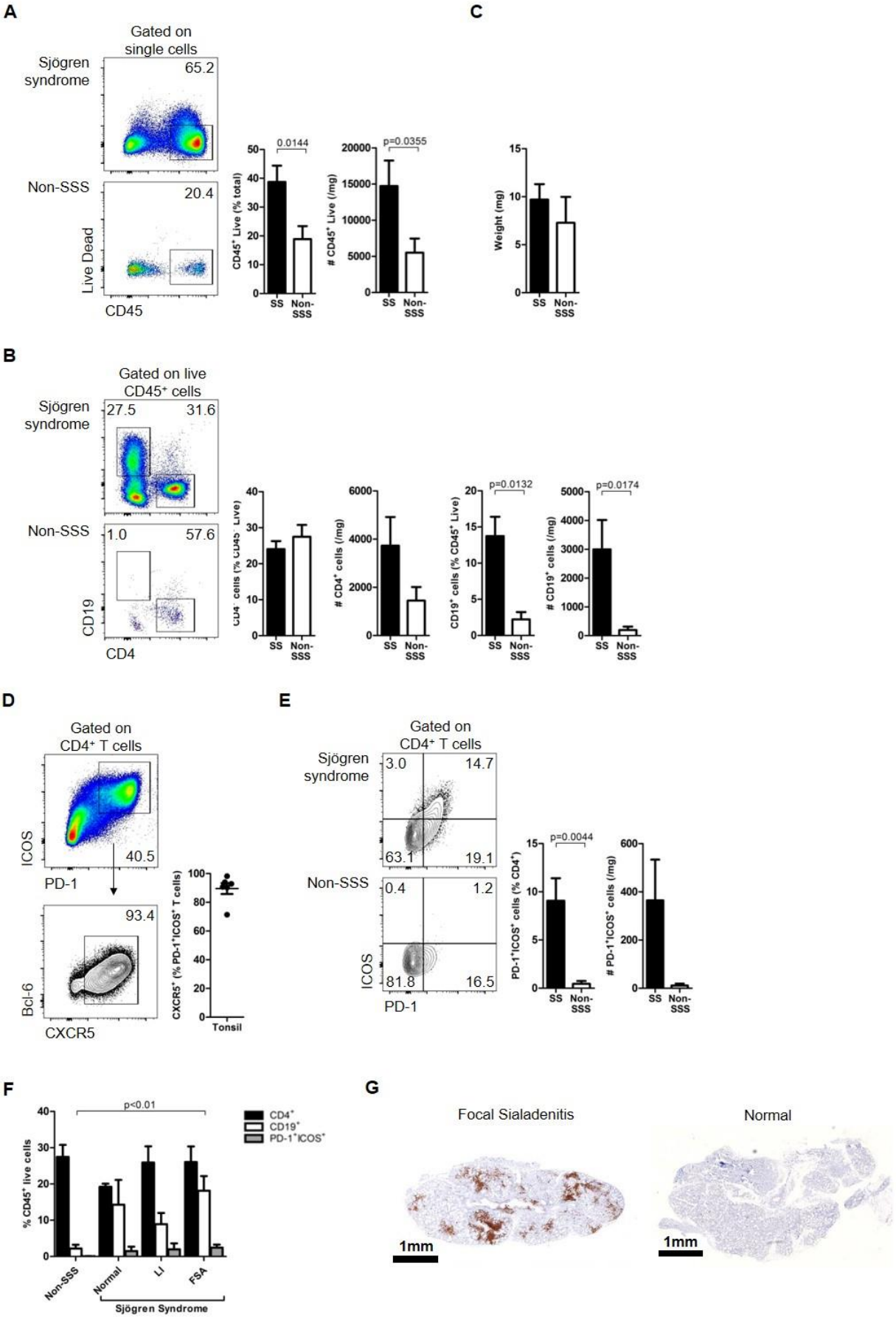
p=0.0180), and $CD19^+$ B cells (r=0.6748, p=0.0462) (**Figure 26A, B**). Moreover, blood Tfr cells were also positively correlated with tissue $CD19^+$ B cells (r=0.6690, p=0.0488) and a positive trend was seen regarding other local populations (**Figure 26A**). Interestingly, despite not significant, blood Tfh cells and Tfh cell subsets negatively correlated with tissue infiltration by all inflammatory cells analysed, showing an opposite trend to blood Tfr cells and Tfr/Tfh ratio (**Figure 26A**). Taken together, these data suggest that blood Tfr cells and Tfr/Tfh ratio indicate pathological lymphocytic infiltration in the target organ of SS.

To test whether increased blood Tfr cells and Tfr/Tfh ratio observed in patients is due to selective exclusion of Tfr cells from FSA lesions we directly accessed $CXCR5^+Foxp3^+$ Tfr cells infiltration in MSG biopsies by microscopy. We found a substantial number of $CXCR5^+Foxp3^+$ Tfr cells within $CD20^+$ FSA in MSG of SS patients (**Figure 26C**). As tissue infiltration by $CD20^+$ B cells is considered the first step of ELS formation[404], our findings suggest that Tfr cells are not excluded from ELS in inflamed SS exocrine glands.

Blood Tfr/Tfh ratio as a marker of SS and focal sialadenitis

As blood Tfr/Tfh ratio was increased in SS patients compared to healthy donors and associated with MSG lymphocytic infiltration we wondered whether this ratio could aid in the identification of patients with SS and FSA, a major diagnostic marker of SS[428,430,442].

Figure 25 (next page): Characteristics of minor salivary gland biopsies of primary Sjögren's syndrome patients.
(A) Frequency and absolute numbers (per mg of tissue) of $CD45^+$ hematopoietic cells in minor salivary gland biopsies (MSG) of SS patients (n=14) and non-Sjögren sicca syndrome (non-SSS) patients (n=6). Representative plots (left) and pooled data (right). Unpaired Student T-test. **(B)** Frequency and absolute numbers (per mg of tissue) of $CD4^+$ T cells and $CD19^+$ B cells in MSG biopsies of SS (n=14) and non-SSS (n=6). Representative plots (left) and pooled data (right). Unpaired Student T-test with Welch's correction for variance. **(C)** Weight of salivary gland tissue removed during MSG biopsy in SS patients (n=14) and non-SSS patients (n=6). **(D)** CXCR5 and Bcl-6 expression by $ICOS^+PD\text{-}1^+CD4^+$ T cells from human tonsils. Representative plots (left) and pooled data (right) (n=6). **(E)** Frequency and absolute numbers (per mg of tissue) of $PD\text{-}1^+ICOS^+$ T cells in MSG biopsies of SS (n=10) and non-SSS (n=6). Representative plots (left) and pooled data (right). Unpaired Student T-test with Welch's correction for variance. **(F)** Frequency of $CD4^+$ T cells, $CD19^+$ B cells, and $PD\text{-}1^+ICOS^+$ T cells in MSG biopsies of SS (n=14) and non-SSS (n=6), according to histological diagnosis (normal, no infiltration by lymphocytes; LI, unspecific lymphocytic infiltration; FSA, focal sialadenitis). Two-way ANOVA with post-test Bonferroni's Multiple Comparison. **(G)** Microscopy of formalin-fixed paraffin-embedded MSG stained for CD20 (brown) in a SS patient with FSA (right) and a SS patient with no pathologic findings (normal, left). Bars on scatterplots represent SEM.


A
Gated on single cells
Sjögren syndrome
65.2
Non-SSS
20.4
Live Dead
CD45
0.0144
CD45+ Live (% total)
p=0.0355
# CD45+ Live (/mg)
SS
Non-SSS
C
Weight (mg)
B
Gated on live CD45+ cells
Sjögren syndrome
27.5
31.6
Non-SSS
1.0
57.6
CD19
CD4
CD4+ cells (% CD45+ Live)
# CD4+ cells (/mg)
p=0.0132
CD19+ cells (% CD45+ Live)
p=0.0174
# CD19+ cells (/mg)
D
Gated on CD4+ T cells
ICOS
40.5
PD-1
93.4
Bcl-6
CXCR5
CXCR5+ (% PD-1+ICOS+ T cells)
Tonsil
E
Gated on CD4+ T cells
Sjögren syndrome
3.0
14.7
63.1
19.1
Non-SSS
0.4
1.2
81.8
16.5
ICOS
PD-1
p=0.0044
PD-1+ICOS+ cells (% CD4+)
PD-1+ICOS+ cells (/mg)
F
p<0.01
CD4+
CD19+
PD-1+ICOS+
% CD45+ live cells
Non-SSS
Normal
LI
FSA
Sjögren Syndrome
G
Focal Sialadenitis
Normal
1mm
1mm

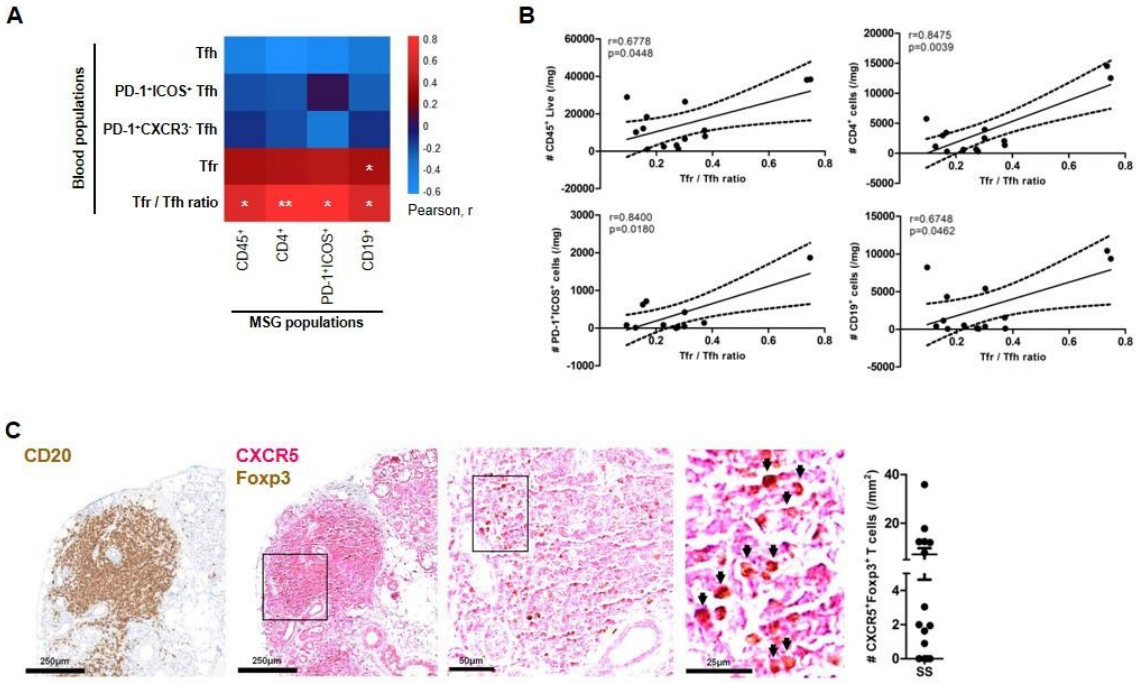


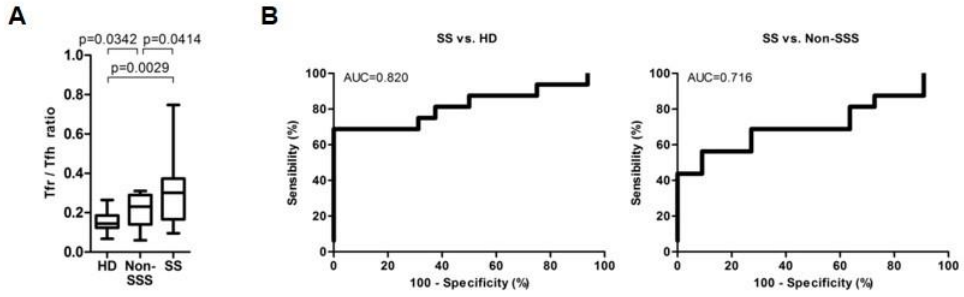
Figure 26: Blood Tfr/Tfh ratio identifies pathological lymphocytic infiltration in Sjögren's syndrome target organ.

(A) Heatmap representation of correlation between peripheral blood Tfh cells, PD-1$^+$ICOS$^+$ Tfh cells, PD-1$^+$CXCR3$^-$ Tfh cells, Tfr cells, and Tfr/Tfh ratio (rows) and salivary gland infiltration by CD45$^+$ hematopoietic cells, CD4$^+$ T cells, ICOS$^+$PD-1$^+$CD4$^+$ T cells and CD19$^+$ B cells (columns), in primary SS patients (n=14, Pearson coefficient, r). **(B)** Correlation between peripheral blood Tfr/Tfh ratio with salivary gland infiltration by CD45$^+$ hematopoietic cells, CD4$^+$ T cells, ICOS$^+$PD-1$^+$CD4$^+$ T cells and CD19$^+$ B cells (n=14, linear regression). **(C)** Identification of CXCR5$^+$Foxp3$^+$ Tfr cells (arrows on extreme right image) within FSA containing CD20$^+$ B cells in MSG biopsies of primary SS patients by immunohistochemistry. Extreme left image shows FSA containing B cells (CD20 in brown) (x100, digital zoom). The three images on right show double immunohistochemistry for CXCR5 (purple/red) and Foxp3 (brown). The squares in each image are amplified successively on the right (x100, x200, x400, digital zoom). Representative sections (left) and pooled data (right) (n=16). Bars on scatterplots represent SEM. On linear regression plots, dashed line represents interpolated 95% confidence interval.

The blood Tfr/Tfh ratio was significantly higher in SS patients compared to healthy donors and patients with non-Sjögren sicca syndrome (**Figure 27A**). This ratio was a significant predictor of SS when compared to healthy donors, with an increase of 4.97 in the odds of having SS for every decimal increase in the Tfr/Tfh ratio (AUC=0.82, p=0.008) (**Figure 27B, C**). Through ROC analysis, we found that a cut-off of ≥0.278 correctly classified 87.1% of patients with 100% specificity and 73.3% sensitivity. Compared to non-Sjögren sicca syndrome, Tfr/Tfh ratio tended to associate with SS diagnosis (OR=1.93, p=0.099, AUC=0.716), with the best cut-off to diagnose SS being a blood Tfr/Tfh ratio ≥0.3009 **(Figure 27B, C**). Thus, patients with sicca symptoms and a Tfr/Tfh ratio ≥0.3009 had 13

times the odds of having SS, with very high specificity (90.9%) but moderate sensitivity (56.3%) (**Figure 27C**).

Importantly, blood Tfr/Tfh ratio was significantly increased in patients with FSA, compared to those with normal histology or mild unspecific inflammation, irrespective of diagnosis (**Figure 27D**). Moreover, it significantly predicted the result of MSG biopsy (FSA vs. no-FSA), with an increase in the odds of having FSA of 2.03 per each decimal increase (AUC 0.793, p=0.047) (**Figure 27C, E**). Values of blood Tfr/Tfh ratio ≥0.302 were significantly associated with FSA (OR=10, p=0.022, AUC=0.757), with good sensitivity (71.4%) and very good specificity (80%) **(Figure 27C**).



C

	OR	p-value	AUR	Sensitivity	Specificity	Correctly classified
SS vs. HD						
Tfr/Tfh ratio*10	4.97	**0.008**	0.820	N/A	N/A	N/A
Tfr/Tfh ratio≥0.2780	N/A	N/A	N/A	68.8	100	84.4
SS vs. non-SSS						
Tfr/Tfh ratio*10	1.93	0.099	0.716	N/A	N/A	N/A
Tfr/Tfh ratio≥0.3009	12.96	**0.028**	0.736	56.3	90.9	70.4
FSA vs. Normal/LI						
Tfr/Tfh ratio*10	2.03	**0.047**	0.793	N/A	N/A	N/A
Tfr/Tfh ratio≥0.3020	10	**0.022**	0.757	71.4	80	77.8

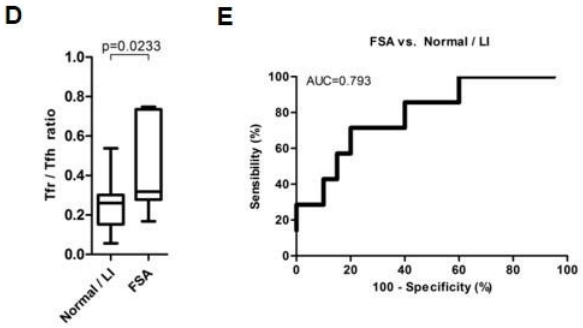


Figure 27: Blood Tfr/Tfh ratio is a marker of primary Sjögren's syndrome and focal sialadenitis.
(A) Blood Tfr/Tfh ratio in Sjögren's syndrome (SS, n=16) and non-Sjögren sicca syndrome (non-SSS, n=11) patients and healthy donors (HD, n =16). Unpaired Student T-test with Welch's correction for variance was used except when variance was not significantly different between

groups (non-SSS vs. HD comparison). One-way ANOVA comparison across groups represented on top. **(B)** Receiver operating characteristic (ROC) curve for prediction of SS diagnosis (vs. HD and vs. non-SSS) based on Tfr/Tfh ratio. AUC, area under the curve. **(C)** Odds-ratio (OR), p-value and AUC of logistic regression models predicting SS diagnosis (vs. HD and vs. non-SSS) and focal sialadenitis (FSA, vs. normal histology or unspecific lymphocytic infiltration, LI) based on Tfr/Tfh ratio as a continuous variable or as specific cut-offs. Tfr/Tfh ratio transformed by 1 decimal place ($*10^1$) for better interpretation of the OR. Percentages of sensitivity, specificity and correct classification of patients based on given cut-offs. **(D)** Blood Tfr/Tfh ratio of patients with sicca symptoms undergoing minor salivary gland biopsy, with focal sialadenitis (FSA, n=7) and normal histology or unspecific lymphocytic infiltration (LI, n=20). Mann-Whitney U test used due to skewed distribution of values and non-different variance according to Brown–Forsythe's test. **(E)** Receiver operating characteristic (ROC) curve for prediction of FSA diagnosis (vs. normal/LI) based on Tfr/Tfh ratio. AUC, area under the curve.

Discussion

Our results show that blood activated Tfh cells and Tfr/Tfh ratio are associated with disease activity and FSA in MSG, respectively, in primary SS. Indeed, there is a striking association between high Tfr/Tfh ratio and ectopic lymphoid activity in salivary glands, the target organ of this disease. In addition, circulating PD-$1^+$$ICOS^+$ Tfh cells seem to provide a different information, indicating disease activity and not FSA. Of note, PD-$1^+$$ICOS^+$ Tfh cells were not increased in primary SS, when patients were analysed together, but this cell population was increased in patients with high disease activity.

The relationship between circulating PD-$1^+$$ICOS^+$ Tfh cells with ESSDAI was an exciting finding. While, many studies have established that circulating activated Tfh cells are correlated with disease severity in autoimmunity[391,454,455], so far circulating Tfh cells were not correlated with ESSDAI. Notably, other Tfh subsets, namely Tfh17-like cells identified in other immune-mediated diseases[391], were not altered in our cohort of primary SS patients. Although, autoimmunity and acute GC reactions induced by vaccination have distinct mechanisms, we anticipated a greater relationship between PD-$1^+$$ICOS^+$ Tfh cells with serum autoantibodies, given the reported increase of this subset following influenza vaccination[120,123]. Considering that the increase of PD-$1^+$$ICOS^+$ Tfh cells following influenza vaccination is only transient, with a peak at day 7 – 10 post vaccination[120,123], we believe that in primary SS there is a chronic sustained inflammatory state (with ongoing GC reactions), which maintains a high frequency of blood PD-$1^+$$ICOS^+$ Tfh cells[330]. This increased inflammatory response will also lead to the clinical and laboratorial manifestations

of primary SS, which are adequately captured by ESSDAI, thus further validating the usefulness of this tool in clinical practice and research. Moreover, these results suggest that $PD1^+ICOS^+$ Tfh cells may be directly involved in the pathogenesis of SS, particularly in those patients with greater inflammatory activity and high ESSDAI scores. A recent study showed that ESSDAI is an independent predictor of lymphoma development in primary SS[456] thus raising the possibility that increased circulating $PD1^+ICOS^+$ Tfh cells are somehow related to the same mechanism that will culminate in hematologic malignancy.

The predictive value of Tfr cells, and especially the Tfr/Tfh ratio, regarding the diagnosis of primary SS and pathological lymphocytic infiltration in salivary glands may appear counterintuitive, given the regulatory role attributed to Tfr cells[257,331]. However, we have recently shown that human blood Tfr cells remain immature and are generated prior to T – B interactions required for acquisition of follicle access and full regulatory function[330]. The increased proportion of Tfr cells in peripheral blood of SS patients did not seem to be due to anatomic exclusion of Tfr cells from inflamed exocrine glands, as substantial numbers of Tfr cells were found within FSA in most patients. Although, we did not perform a detailed characterization of FSA regarding their organization as ELS, all patients with FSA had organized clusters of $CD20^+$ B cells within those lesions. As tissue infiltration by $CD20^+$ B cells is considered the first evidence of ELS development[404], we assumed that patients with $CD20^+$ FSA had ELS. It is generally assumed that ELS are populated by lymphocytes formed outside these structures, in secondary lymphoid organs[356,357]. Thereby, it is likely that Tfr cells result from enhanced differentiation in secondary lymphoid organs due to ongoing humoral responses. Our data suggest that accumulation of Tfr cells are not sufficient to restore tolerance in exocrine glands (where the first step of SS pathogenesis occur), although we did not directly address the presence of Tfr cells in ELS with and without GCs[439]. A recent report claimed salivary gland epithelial cells induce Tfh cell differentiation from naïve $CD4^+$ T cells[407]. While, it is not known whether Tfr cell differentiation can also occur *in situ,* this could possibility provide an alternative explanation for the increased proportion of blood Tfr cells in SS.

The blood Tfr/Tfh ratio does seem to be a specific of ELS formation within salivary glands. Indeed, we found that Tfr/Tfh ratio could discriminate between SS patients and healthy donors with excellent accuracy, with a specific cut-off above which the diagnosis of SS was almost certain. Even more important, this ratio was also helpful in predicting a diagnosis of

primary SS and the presence of FSA in MSG biopsy in a group of patients investigated for sicca symptoms, thus highlighting the potential clinical value of this marker. The proposed cut-offs of blood Tfr/Tfh ratio above/equal 0.3009 and 0.302 were strong predictors of SS and FSA, respectively, and constitute promising tools for SS clinical evaluation. It should be noted that the specificity and sensitivity of Tfr/Tfh ratio to predict FSA and SS was remarkably high, in particular given the small number of patients studied. We anticipate that greater patient numbers may reinforce the significance of our findings, potentially establishing the usefulness of Tfr and Tfh quantification for primary SS patient stratification. This is particularly important both for diagnostic and therapeutic purposes as there are major unmet needs in these fields in primary SS[457]. The potential to confirm diagnosis in patients who do not fulfil classification criteria, or to identify clusters of patients more prone to respond to targeted therapies are some of the applications that such a biomarker could have.

For the results described in this section, we decided to study fresh blood of patients at the time of diagnosis who were not under immunosuppressant drugs, to overcome potential drug effects on Tfh and Tfr cell subsets. Consequently, the studied cohort of patients was small, but uniform and consistent. Other limitations of our study included the fact that paired samples for every parameter were, in some cases, not available and the technical difficulty in achieving CXCR5 staining in salivary gland cell suspensions, which precluded a direct assessment of tissue Tfr cells at a phenotypical and functional level. In addition, we did not dissect the organizational complexity of FSA, namely, the presence of high endothelial venules, development of $CD21^+$ FDC networks, infiltration by $CD138^+$ plasma cells, and the expression of GC-specific proteins such as Bcl-6 and AID[357]. It also remains to be shown whether alterations of Tfr and Tfh subsets that we describe here are specific for SS or can be generalized to other immune-mediated inflammatory diseases, in particular diseases with ELS and autoantibody-mediated pathology.

CHAPTER 5

GENERAL DISCUSSION

Provision of improved hygiene conditions, campaigns of mass vaccination, and eradication of mass epidemics were the greatest medical achievements of twentieth-century. The twenty-first-century medicine is facing new challenges. Modernity inspired people to become more demanding, and to hardly accept disappointing results. Even if etiology is well-known, new therapies will keep missing the opportunity to directly target disease initiators (other than infectious agents), until disease prediction becomes a universal practice. Dysregulated immune responses are currently recognized as a cornerstone of disease perpetuation. Thus, targeting the immune system to modulate disease, without affecting immunocompetence, is probably one of the biggest challenges of modern medicine.

Although, the immune system has an inbuilt tendency to avoid attacking self-tissue, as proposed by Paul Ehrlich in 1901, aberrant responses to self do occur[458]. Half century later, Sir Frank Burnet reasoned that somatic mutations in antigen receptors leading to "forbidden" clones that were mistakenly not deleted during lymphocyte development can give rise to autoimmune diseases[427,459]. The ability to reprogram the dysregulated immune system towards reinstating homeostasis without the need for continuous treatment is the holy grail of immunotherapy. Conventional therapies for autoimmunity rely primarily on harsh broad-spectrum anti-inflammatory and suppressive regimens. The adverse effects of such regimens have driven the continuous quest for more specific and less toxic therapies. In the last 20 years, the use of immunological tools, such as monoclonal antibodies, receptor-immunoglobulin fusion proteins, vaccines and immune cells, as therapeutic options improved our ability to tread the narrow line between treatment efficacy and unacceptable collateral damage[358]. Because immunotherapy manipulates the fundamental components of the immune response that underlies both the pathological and the normal immune functions, penalty can probably never be entirely separated from success. In addition to the associated hazards of immunotherapy, the failure of most immunological approaches that are effective in animal models to modulate autoimmune diseases in humans suggests that we do not understand many of the principles behind pathogenic mechanisms of those diseases[358]. To minimize the unintended side effects of immunotherapy, we must develop more specific methods to target pathogenic cell populations and to identify new disease biomarkers. Indeed, as we gain more experience, we are learning to use existing therapies in better ways and developing treatments with better efficacy-to-toxicity ratios. Many options are now being studied, such as using a combination of therapeutic agents to reduce the dose of each individual drug to levels that are not toxic, and to start treatment at early time points, given

that a shorter course of treatment is expected to reduce the side effects[460]. Moving from disease biomarkers to biomarkers of specific abnormal immune reactions may be a breakthrough to timely treat autoimmune patients with the most appropriate therapeutic regimens.

Autoimmunity of follicular origin poses an additional challenge because the therapeutic options need to "tune down" GC reactions to reduce the risk of autoimmunity while preserving foreign antigen-specific protective responses. Following the discovery of Tfr cells as putative GC "fine tune" regulators, targeting Tfr cell responses may constitute a novel and highly selective approach for the treatment of autoantibody-mediated autoimmune diseases. Yet, the still embryonic knowledge about Tfr cell biology halts these advances, particularly in humans.

The main topic of this thesis is human Tfr cells. The overall aim of this work was to study human blood CXCR5$^+$Foxp3$^+$ Treg cells from two perspectives. First, we conducted several experiments to address whether CXCR5$^+$Foxp3$^+$ T cells constitute a circulating counterpart of germinal centre *bona fide* Tfr cells. While, these cells increased after the induction of GC responses by vaccination and are absent from neonatal cord blood (an immune setting in which GC reactions do not take place), confirming their relationship with humoral responses in secondary lymphoid organs, they fail to fully regulate humoral responses, suggesting these cells are not fully competent Tfr cells. These findings may be translated therapeutically as far as modulation of blood Tfr cells toward proficient Tfr cells can be experimentally achieved. Second, we found that blood Tfr cells, and specifically the Tfr/Tfh ratio, are increased in SS patients. Notably, patients with ectopic lymphoid activity have the highest level of blood Tfr/Tfh ratio. These findings led us to propose blood Tfr/Tfh ratio as a novel biomarker of ectopic lymphoid activity in SS.

Human Blood CXCR5$^+$Foxp3$^+$ Treg Cells Are Immature Tfr Cells Not Fully Licensed with Humoral Suppressive Function

Our comprehensive evaluation of human Tfr cells supports a model in which blood Tfr cells are generated following the initial steps that lead to GC responses in secondary lymphoid tissues, exiting the tissue prior to interactions with B cells that are required for complete

differentiation towards tissue resident Tfr cells (**Figure 20A**). Our work was the first to address Tfr cell biology and ontogeny in humans. Having demonstrated that Tfr cells are a distinct subset of both Tfh and (conventional) Treg cells, we believe human T cell immunophenotyping studies will become clearer from now on. Moreover, we showed key differences between mice and humans regarding the function of blood Tfr cells: while murine blood Tfr cells appear to be specialized in suppressing antibody production (despite their lower suppressive capacity when compared to tissue Tfr cells), human blood Tfr cells do not have the ability to fully suppress humoral responses. Therefore, our findings also advert for the limitations of translating mice knowledge to human therapeutics in this field. Animal models have been massively used in life sciences research due to their experimental advantages. Their use allows a rigorous and independent study of experimental variables and access to tissues not ethically accessible in humans. Yet, several immunological approaches that are effective in animal models have failed in humans[358]. We will probably face a turnover in this scientific paradigm in the years to come: instead of using animal models to explore new hypothesis, the use of human samples to generate new clinically-relevant hypothesis (to be further validated in animal models) will be standard. The adaptive immune responses may have critical distinctions between humans and mice[322,461,462]. For example, human Tfh cells require IL-23, IL-12 and TGF-β for their differentiation, in contrast to IL-6 requirement for murine Tfh cells[63,126]. Interestingly, the impact of bystander infections on basal immune system activation (and on Tfh, Tfr and GC B cell responses, which are anticipated to be highly mediated by pathogens) may account for differences reported between murine and human immune system[461].

The absence in blood Tfr cells of Bcl-6, ICOS and PD-1 expression (which was also reported for human blood Tfh cells[52]) together with their ineffectiveness to fully suppress humoral responses can be argued as sufficient evidence to disprove human blood $CXCR5^+Foxp3^+$ T cells as circulating counterparts of *bona fide* tissue Tfr cells. However, we found that blood Tfr cells specifically migrated toward a CXCL13 gradient, while TCR/CD28 stimulation alone was not sufficient to induce CXCR5 upregulation in conventional Treg cells. Together with the complete absence of $CXCR5^+Foxp3^+$ T cells in neonatal cord blood (where activated $CD45RO^+ICOS^+$ Treg cells were already present) led us to conclude that CXCR5 expression is acquired in secondary lymphoid tissue after establishment of mature humoral responses, and not simply an activation marker. This conclusion is further supported by the immature phenotype of blood Tfr cells and their emergence prior to B-cell interactions, as

B-cell deficient patients had normal frequencies of blood Tfr cells. Thereby, the key question turns out to be *what is the biological relevance of these cells in human blood*? Or, alternatively, *why Tfr cells leave secondary lymphoid organs before having the opportunity to exert their function within GCs*?

In murine models, adoptively transferred blood Tfr cells have been shown able to migrate to secondary lymphoid organs with ongoing GC reactions and to the skin[258]. In mice parabiosis studies, circulating Tfr cells were reported to have memory properties and to outcompete *de novo* formed effector Tfr cells after secondary exposure to antigen[258]. These findings suggest that a major role of blood Tfr cells can be the regulation of secondary GC reactions, raising the threshold for B cell activation, thereby limiting inappropriate antibody responses. While, these results do not explain the immature phenotype of human blood Tfr cells, they support a model in which Tfr cells are formed upon GC-forming immune reactions. Indeed, we demonstrated that human blood Tfr cells increase at day seven following influenza vaccination. So far, it has been reported that: a) human blood $ICOS^+CXCR5^+$ Tfh cells peak at day seven upon influenza vaccination and correlate with specific protective antibodies[120,123,463]; b) human blood $CXCR5^+$ Tfh cells are memory-like cells which promote antibody responses upon antigen reexposure[52,54–56]; and c) in mice, lymph node Bcl-6^+CXCR5^+ Tfh cells peak between day 7 and day 15 upon intranasally influenza infection, but Bcl-6^+CXCR5^+ Tfr cells only peak at day 30[278]. Together with our data, it seems tempting to hypothesise that Tfh and Tfr cells follow distinct kinetic responses upon influenza vaccination: while Tfh cells accumulate in blood and lymph nodes almost simultaneously, recirculating as GC-experienced memory Tfh cells, Tfr cells accumulate in lymph nodes only after systemic recirculation as immature cells. Whether all Tfr cells follow this kinetic profile, or only some cells recirculate is still unknown. We may speculate that early recirculation of Tfr cells following adaptive immune responses enforces systemic tolerance by unknown mechanisms.

The therapeutically use of Tfr cells to re-establish immune tolerance by fine tuning GC reactions is an attractive and promising idea. While, several studies linked the absence of Tfr cells to the emergence of autoimmunity in murine models, we still do not understand many fundamental aspects of Tfr cell biology. The impact of Tfr cells on the immune system is hardly consensual. In different murine models, absence of Tfr cells led to expansion of Tfh and GC B cells, as well as increased antibody production[256,254,255,278]. However, some

other groups have shown that the lack of Tfr cells has no impact on GC size and GC B cell numbers[303]. The effect of Tfr cells on antigen-specific B cells and antibody affinity is even more complex. Existing evidence suggests that Tfr cells restrains generation of antibodies specific to the immunizing antigen while favouring the emergence of B cell clones secreting high affinity antibodies[259,269,278,302,303]. Thus, Tfr cells impose (on GC B cells) a more stringent competition for Tfh help, promoting the generation of high affinity antibodies, while limiting the expansion of antigen specific B cell clones. As Tfr cells seem to uncouple GC B cell expansion and selection, it would be interesting to address the impact of Tfr cells on B cell cycling between GC dark and light zones. However, this uncoupling effect by Tfr cells may be time-dependent, as suggested by Wing and colleagues[269]. Further studies using specific Tfr cell deletion, or boosting Tfr numbers, at different time points will be critical to directly address this issue. It would also be interesting to address the impact of Tfr cells on autoreactive self-sustained GC responses, which were recently proposed as the underlying mechanisms to epitope spreading observed in autoimmune diseases[464]. Degn and colleagues used the 564Igi murine model (generated by knock in of the heavy chain and kappa light chain of an autoreactive B cell clone targeting ribonuclear complexes) to demonstrate that a single autoreactive B cell clone drives a TLR7-dependent expansion of other autoreactive B cells in spontaneous GCs[464,465]. Those GC gained independence from the initial 546Igi trigger, but evolved toward pauciclonality[464]. Importantly, these autoreactive GCs were ablated by CD40L blockade, suggesting that while autoreactive GCs became independent of the initial clone (thereby propagating the disease), they still require T cell help[464].

It is likely that boosting Tfr cell responses will facilitate the emergence of high affinity B cells clones toward the immunizing antigen. Importantly, this effect may be achieved independently of Tfr cell antigen-specificity, as Tfr cells were shown to suppress in an antigen-independent manner[258,264]. It is also possible to anticipate that boosting Tfr cell responses will not culminate in gut dysbiosis, as Tfr cells were demonstrated to be required for gut IgA production and microbiota homeostasis[302]. In the setting of autoimmunity, a protective function has been ascribed to Tfr cells. In the absence of Tfr cells, some murine models developed clinical hallmarks of autoimmunity, such as the production of clinically relevant autoantibodies (anti-dsDNA, anti-Ro52, anti-Ro60 and anti-La) and autoantibody-induced glomerulonephritis[273,278,282,283]. It is notable that under those conditions, the absence of Tfr cells alone appears to be sufficient to induce autoantibody-mediated autoimmunity, as several redundant mechanisms maintaining immune tolerance within GCs have been

described[143]. Disappointingly, no study has specifically tested whether the adoptive transfer of Tfr cells control ongoing autoimmune diseases. In chronic graft-versus-host disease, an autoantibody-mediated disease, CXCR5-expression by Treg cells was shown to be required for their protective effect, but the therapeutic potential of Tfr cells remains purely speculative[308].

Before testing Tfr cell-based immunotherapy in humans it is critical to address whether human tissue Tfr cells are intrinsically capable to regulate humoral responses (like their mice counterparts). We were unable to sort pure Tfr cells from human tonsils precluding the *in vitro* assessment of their function and a direct comparison between blood and tissue Tfr cells. Indeed, only a minority of sorted $CXCR5^{+}ICOS^{+}CD25^{+}CD127^{-}CD4^{+}$ (the gate used for Tfr cells) tonsil cells expressed Foxp3 (**Figure 18**). This can be due to contamination by $CD25^{+}$Bcl-6^{lo} Tfh cells. In human tonsils, a $CD25^{+}$Bcl-6^{lo} Tfh cell population, expressing IL-21 and c-Maf, was reported to provide help to B cell in a IL-2-dependent manner[466]. Whether this $CD25^{+}$Bcl-6^{lo} Tfh cell population is unique to human tonsil microenvironment or systemically relevant is not known. While, these findings suggest a role for IL-2 signalling in GC responses, robust data demonstrated that IL-2 signalling inhibits murine Tfh cell differentiation and GC formation[83,85,279]. Additionally, recent studies claimed that the most mature Tfr cells express low CD25 to sustain the Bcl-6-dependent Tfr cell program[276–278]. In this respect, the usage of a stringent $CD25^{hi}$ sorting strategy can be argued as another reason for the poor suppressive function of our sorted Tfr cell population. Thus, alternative approaches are required to efficiently sort $Foxp3^{+}$ Tfr cells from human tissues. New Tfr cell markers can be tested and different tissues can be used. Our group is now studying new sorting strategies for human tissue Tfr cells using, not only new Tfr cell markers (such as IL-1R2), but also other secondary lymphoid organs. IL-1R2, the IL-1 receptor decoy, was recently reported to discriminate Tfr cells from Treg and Tfh cells in murine studies[277]. Interestingly, IL-1 strongly increases B cell proliferation and induces CD40L and OX40 expression by T cells, thereby favouring B-T cell interactions in adaptive immune responses[467–469]. The sequestration of IL-1 by IL-1R2-expressing Tfr cells can be hypothesised as one additional regulatory mechanism of this Treg cell subset. Although, Tfr cells need a balanced Bcl-6- and CD25/IL-2/Foxp3-dependent program, it remains to be demonstrated whether there are two subsets of Tfr cells based on CD25 expression, or whether true Tfr cells lack CD25 expression[276–278]. Moreover, no studies yet accessed *in situ* CD25 expression by human GC Tfr cells. The most attractive model to explain the apparent

paradox of those two cellular programs was recently proposed by Botta and colleagues. They showed that Tfr cells loose CD25 expression throughout their differentiation pathway toward fully differentiated GC-resident Tfr cells[278]. This data is consistent with our model in which blood Tfr cells emerge from secondary lymphoid tissues prior to B cell interactions, thereby being immature (not fully differentiated) $CD25^+$ Tfr cells.

Blood is the most accessible source of human immune cells, prompting another question: *how to induce full differentiated Tfr cells from immature blood Tfr cells*? Tfr cell differentiation has classically been studied in a Tfh cell-biased manner rather than as a unique process. Current knowledge has established significant differences between the Tfh and Tfr cell differentiation pathways. Specifically, PD-1 and CTLA-4 signalling selectively affect Tfr cell differentiation; IL-21 seems to orchestrate a feedback control mechanism in humoral responses, favouring Tfh cell differentiation and function while restraining Tfr cell differentiation; and mTORC signalling differently affect Tfh and Tfr cell differentiation (while IL-2/mTORC1 signalling axis seems to inhibit Tfh cell differentiation, mTORC2 was reported to be essential for Tfh cell differentiation)[259,260,269,270,272,273,289]. Further research must now chase detailed requirements for full Tfr cell differentiation. First, the existence of a thymic Tfr cell pre-committed Treg cell subset needs to be addressed. Then, it will be important to study the antigenic and environmental cues required to induce Tfr cell differentiation from the thymic Treg cell pool. Single-cell RNASeq-based technologies are revealing a much greater heterogeneity in immune cell populations[470]. Therefore, the absence of follicular markers on thymic Treg cells at the protein level should not be used to exclude the possibility of thymic Tfr cell pre-committed Treg cells. On the contrary, the use of those technologies may aid in the study of Tfr cell differentiation in human tissues. By studying Tfr cells at the single-cell level the evolving transcriptomic changes will be known. This knowledge may be used to find candidate genes responsible for the Tfr cell differentiation pathway (including its environmental cues) as well as to address whether a Tfr cell program is hidden in some thymic Treg cells.

All the described murine studies reported an expansion of (tissue) Tfr cells following immunization. In humans, we also demonstrated that blood Tfr cells increase upon influenza vaccination. Inflammatory signals augment the differentiation of activated Treg cells from resting Treg cells, possibly by enhancing antigen presentation and supplying T cells with accessory signals of activation[27,280,471–473]. Therefore, it is likely that the inflammatory milieu

induced by immunization favour the expansion of Tfr cells. Whether this expansion is mediated by self or foreign antigens is not known. Although, it is commonly assumed that Treg cell TCR repertoire is skewed toward recognition of self-antigens, several studies challenged this assumption, demonstrating that TCR is highly diverse[174,474]. Our group showed that although Tfr cells undergo proliferation, their TCR is not specific for the antigen driving the GC reaction since the TCR usage has little in common with Tfh cells repertoire (but closely resembled the self-skewed TCR repertoire of thymic Treg cells)[264]. On the contrary, under certain conditions, Tfr cells were showed to be specific for the immunizing antigen[263]. However, even under those conditions, antigen-specific Tfr cells did not seem to overcome the need of Tfr cells derived from thymic Treg cells. Interestingly, mice immunized with insulin (self-antigen) had a higher proportion of tissue Tfr cells (compared to OVA immunization)[277]. Although it is established that, like Treg cells, Tfr cells exhibit a TCR-non-specific bystander suppression of effector T cells, further studies should clarify the nature of Tfr cell driving antigens, as well as the cytokines required for Tfr cell expansion and maintenance, before addressing the methods to modulate Tfr cell responses[258,475]. A thorough study of Tfr cell TCR repertoire (using new computational biology approaches) may help defining antigen candidates for driving the differentiation of this cell population. Several cytokine combinations may be also tested for *in vitro* induction of Tfr cells from conventional $CXCR5^-$ Treg cells.

IL-2 was recently proposed to act as a rheostat during physiological GC responses induced by viral infections[278]. Upon influenza infection, expansion of Treg cells consumed IL-2 from lymph node microenvironment facilitating virus-specific Tfh cell responses. The increased IL-2/CD25/STAT5 signalling on Treg cells prevented their differentiation toward a Tfr cell fate (through Bcl-6 inhibition). As IL-2 signalling decreased during the time course of influenza infection, Treg cells undergone a Bcl-6-dependent Tfr cell differentiation pathway. Finally, Tfr cells expansion prevented the emergence of self-reactive B cell clones formed during infection-induced GC reactions. These results suggest that blocking CD25 may lead to augmented Tfr cells responses. However, the impact of IL-2 on different T cell populations is extremely complex: IL-2 is essential for the development and maintenance of Treg cells, as well as for inducing terminally differentiated effector (Th1 and Th2) T cells, whereas strong IL-2 signals inhibit Tfh cell diffentiation[83,206]. Indeed, the impact of IL-2 on Treg and Tfh cells in patients with ongoing autoimmune diseases in not straightforward. In a small study, low-dose IL-2 treatment was effective in reducing disease activity in SLE

patients. During the 12-week period of this prospective study, a decrease in blood Tfh and Th17 cells was observed along with an increased frequency of blood Treg cells[476]. Whereas, Tfr cells were not directly analysed, the results of this study highlight the complexity of IL-2 axis in disturbed autoimmunity-prone adaptive immune responses.

Blood Tfr/Tfh Ratio Marks Ectopic Lymphoid Structure Formation in Primary Sjögren Syndrome

We have shown that blood activated Tfh cells and Tfr/Tfh ratio are associated with distinct aspects of primary SS pathogenesis. While, high blood Tfr/Tfh ratio is correlated with the presence of FSA in salivary glands of SS patients (the target organ of this disease), circulating PD-1^{+}ICOS^{+} activated Tfh cells indicated SS disease activity (but not ELS formation).

The predictive value of blood Tfr cells, and especially the blood Tfr/Tfh ratio, regarding the diagnosis of primary SS and the identification of pathological lymphocytic infiltration in salivary glands may appear counterintuitive. First, Tfr cells were described as regulators of GC responses, thus decreased Tfr/Tfh ratios (in both secondary lymphoid organs and blood) have been associated with augmented autoantibody production by GC B cells[257,260,297,331]. Second, IL-21 has been reliably implicated in the development of SS[477]. The reported impact of IL-21 on Tfr cell differentiation and proliferation would predict a decreased proportion of Tfr cells in IL-21-drived conditions[270,272,273].

In systemic autoimmune conditions with disturbed and chronic GC reactions we anticipated that Tfr/Tfh ratio would be decreased due to an unproportioned increase in Tfh cells[143]. Consistently, blood Tfr cells were found reduced in the blood of MS, SLE and MG patients[329,341,346]. However, these studies used different markers to identify blood Tfr cells and found unreliable relationships between blood Tfr cells and disease activity, autoantibody production, and treatment regimens. For example, blood Tfr cells were increased in patients with AS[338]. On the other hand, our observations were done following a comprehensive study of human Tfr cells and on a cohort of immunosuppressant drug-naïve patients. Given that blood Tfr cells emerge prior to B-cell interactions and only following mature adaptive immune responses, the increased proportion of Tfr cells in peripheral blood of primary SS

patients may be a consequence of ongoing GC reactions. Indeed, the increased proportion of activated PD-1^{+}ICOS^{+} Tfh cells observed in our SS cohort support the existence of ongoing GC reactions in these patients, as those cells were found to correlate with humoral responses induced by vaccination[120,123].

The current theory explaining the pathogenesis of SS (also termed "autoimmune epithelitis") suggests that salivary gland epithelial cells (SGEC) are central regulators of the autoimmune response by acting as atypical antigen-presenting cells and by constitutively expressing a plethora of immune-competent molecules, which are implicated in lymphoid cell recruitment, homing, activation, differentiation, and proliferation[439,451,478–481]. Although, the trigger for altered SGEC remains speculative, the microenvironment changes induced by SGEC lead to development of ELS, *in situ* autoantibody production, and subsequent epitope spreading[420,439,451,481,482]. Recent evidence supports this hypothesis, as once tolerance has been broken for one self-antigen, autoreactive GCs targeting other self-antigens can be spontaneously generated[464]. Whether Tfr and Tfr cells responses in SS are initiated in inflamed exocrine glands or in secondary lymphoid organs is still unknown. While, it is generally assumed that ELS are populated by lymphocytes formed outside these structures, in secondary lymphoid organs, one study reported that SGEC activated CD4^{+} T cells *in vitro* and mediated their differentiation into Tfh cells[356,357,407]. We observed *in situ* Foxp3^{+}CXCR5^{+} Tfr within FSA lesions enriched for CD20^{+} B cells in SS patients, suggesting anatomic exclusion of Tfr cells from inflamed exocrine glands cannot explain the increased proportion of this T cell population in peripheral blood of SS patients. In our work we hypothesized that CD20^{+} FSA harboured typical ELS responses, as tissue infiltration by CD20^{+} B cells was shown to occur in the first steps of ELS development[404]. However, we did not dissect ELS-defining features in FSA , such as segregation of T cells and B cells into discrete areas, development high endothelial venules, differentiation of networks of FDC, accumulation of CD138^{+} plasma cells, as well as the *de novo* ectopic expression of genes encoding proteins, such as AID and Bcl-6[356,357]. Nevertheless, it is likely that *Tfr cell expansion, either in secondary lymphoid organs or in ELS, occurs in primary SS pathogenesis*. This is further supported by the observation that absolute number of Tfr cells in peripheral blood of SS patient did not follow the trend imposed by their lymphopenic state. Indeed, the absolute number of blood Tfr cells did not differ between SS patients and healthy donors, while all other tested T cell populations were decreased (as measured by absolute numbers per mL of blood) in agreement with lymphopenia.

To unify our findings with current knowledge of SS pathogenesis one may argue that Tfr cells expand as an (insufficient) attempt to restore immune tolerance. Different murine models have demonstrated that CTLA-4 deletion led to an increased frequency and absolute numbers of Tfr cells (in lymph nodes as well as in blood)[259,269]. These mice developed spontaneous GCs and T-cell-mediated autoimmunity, suggesting that expansion of Tfr cells can occur concomitantly with autoimmunity, as far as Tfr cells are defective in CTLA-4-mediated GC regulation[259,269]. In humans, monogenic defects involving CTLA-4 and its intracellular trafficking regulator (LRBA, lipopolysaccharide-responsive beige-like anchor protein) lead to a primary immunodeficiency characterized by recurrent infections and autoimmunity (manifested by inflammatory bowel disease, autoimmune endocrinopathies, and cytopenias)[483–489]. Exaggerated Tfh cell responses were recently linked to failure of CTLA-4-mediated regulation in CTLA-4 and LRBA deficiencies[487,490]. In these patients, treatment with Abatacept was found to, at least partially, restore immune homeostasis[490]. Abatacept is a fully human fusion molecule of CTLA-4 with IgG-Fc, that binds to CD80/86 and consequently impairs CD28-mediated T cell co-stimulation. To date, two open-label studies have evaluated the efficacy of Abatacept in primary SS patients[491,492]. Adler and colleagues observed histological, cellular and serological changes in response to treatment, including a decrease in local Treg cells and serum gammaglobulins[492]. It is noteworthy, that treatment with Abatacept induced a decrease in salivary gland Treg cells. Indeed, other studies found that Treg cells were increased in salivary glands from SS, even though these tissues harbour dysregulated autoimmune responses[493,494]. Meiners and colleagues showed that Abatacept improves systemic disease activity and patient-reported symptoms, measured by ESSDAI and Patient Reported Index (ESSPRI), respectively[491]. The effects of Abatacept on human T cells and on T cell-dependent B cell hyperactivity in primary SS where further analysed by Verstappen and colleagues[495]. In this recent report, Abatacept reduced the number, proportion and activation (assessed by PD-1 and ICOS expression) of circulating Tfh cells, as well as serum levels of IL-21, serum titres of autoantibodies (anti-Ro52, anti-Ro60, and anti-La), and number and proportion of blood $CD27^{+}CD38^{+}$ plasmablasts[495]. It may be hypothesized that a *selective defect in CTLA-4-mediated Tfr cell function is acquired during SS pathogenesis*. Alternatively, an aberrant CXCR5 expression intrinsic to Treg cells may explain our findings, as variants in the CXCR5 region have been associated with SS in genome-wide association studies[496].

Many studies have established a strong IL-21 signature in primary SS pathogenesis[477,497–499]. Given the reported role of IL-21 on Tfr cell biology it would be anticipated a decrease proportion of Tfr cells in SS patients[270,272,273]. Jandl and colleagues claimed that a decrease IL-2 responsiveness, mediated by IL-21-dependent Bcl-6 upregulation (which in turn decrease CD25 expression), underlie the Tfr cell inhibition[270]. However, the impact of IL2/IL-21 axis on Tfr cells (and on immune systems) seems far more complex[278,500]. If a decrease CD25 expression on Treg cells was responsible to limit Tfr cell differentiation, as suggested by Jandl and colleagues, CD25 downregulation could not be argued as a consequence of Tfr cell differentiation, as demonstrated by Botta and colleagues[270,278]. Moreover, recombinant IL-2 treatment would hardly inhibit Tfr cell differentiation, as Botta and colleagues also showed[278]. Importantly, it is still not known whether human Tfr cells express IL-21 receptor. Thus, the strong IL-21 signature underlying SS pathogenesis cannot be used to overturn our observations. The further assessment of IL-2/IL-21 axis and of CTLA-4-mediated Tfr cell responses (together with a detailed characterization of salivary gland infiltrating Tfr cells) in SS will probably be of great clinical value. To specifically address infiltrating Tfr cells in non-lymphoid tissues new methodological approaches will be needed to circumvent the technical limitations with conventional methods.

Consequently, one imperative question is *whether blood Tfr cell expansion is a specific feature of SS or a common feature of autoimmune diseases*. Our group is now addressing this specific issue on distinct human autoimmune conditions, from organ specific Hashimoto's thyroiditis to systemic diseases, such as RA and SLE. One possibility is that blood Tfr cells are increased in autoimmune diseases in which ELS formation plays an important pathogenic role[356,357]. Indeed, we found that the blood Tfr/Tfh ratio does seem to be a specific marker of ELS formation within salivary glands.

From a clinical perspective, our findings may aid in decomposing SS heterogeneity. Within a single autoimmune disease, there is considerable variation in clinical manifestations and severity[427]. It is widely accepted that disease heterogeneity is probably the result of intricate genetic and non-genetic factors which account for subtle differences in immunopathogenic mechanisms. The unpredictable response to target therapies both at disease and patient level further highlights autoimmunity heterogeneity. A detailed and global understanding of the human immune system could help to identify individual *immunotypes*[501]. For example, Banchereau and colleagues longitudinally profiled the blood transcriptome to assess SLE

molecular heterogeneity[502]. This analysis enabled patient stratification into seven groups and provided an explanation for the failure of clinical trials, as SLE patients are highly heterogeneous[502]. Our results suggest that primary SS can be stratified into four groups: a) patients with high disease severity scores (measured by ESSDAI) and increased blood activated PD-1^{+}ICOS^{+} Tfh cells, b) patients with FSA and increased blood Tfr/Tfh ratio, c) patients with high disease severity scores and FSA, and d) patients without any of these features (**Figure 28**). Autoimmune epithelitis is only one possible explanation for the immunopathology of primary SS. Several studies have also suggested role for neuroendocrine mechanisms that is related to the influence of hormones and neuropeptides in the function of the exocrine glands[503]. These alternative mechanisms could explain why some patients with primary SS present with severe sicca symptoms with no inflammatory histological features. Interestingly, very recently, some human Tfh cells were found to contain chromogranin marked granules, normally found in neuronal presynaptic terminal storing catecholamines[504]. Whether neuroendocrine dysfunction have an impact on ELS formation and Tfh cell responses in autoimmunity is not known. While, we did not address the impact of disease duration in Tfh cell subsets and Tfr cells, the identification of a specific pathogenic mechanism (namely "autoimmune epithelitis") by Tfr/Tfh ratio could be a major step in achieving earlier and targeted therapies in primary SS. Importantly, so far, no formal correlation was demonstrated between ESSDAI and histopathological findings on salivary gland biopsies[436,505]. However, FSA severity (assessed by focus score) is an important predictor for lymphoma development in these primary SS[428,442].

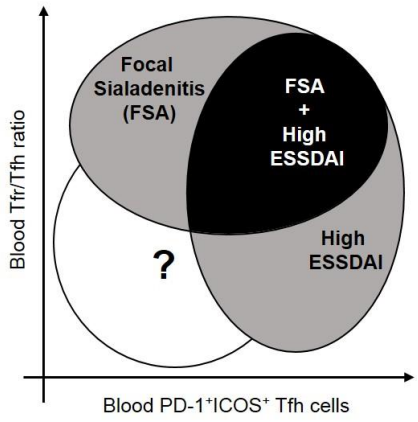


Figure 28: Proposed model for primary Sjögren's syndrome patient stratification
Disease severity (measured by ESSDAI), focal sialadenitis (FSA) on MSG biopsy, activated blood Tfh cells and blood Tfr/Tfh ratio define four subgroups of primary Sjögren syndrome patients: a) patients with high ESSDAI scores and increased blood activated PD-1^{+}ICOS^{+} Tfh cells, b) patients

with FSA on MSG biopsy and increased blood Tfr/Tfh ratio, c) patients with both disease signatures, and d) patients without any of these features

Overall, our findings suggest that immunopathogenic stratification of SS patients could be achieved through quantification of blood Tfh and Tfr cell subsets. Blood Tfr/Tfh ratio seems to help in predicting the presence of FSA in target tissues of SS pathogenesis. We propose the use of blood Tfr/Tfh ratio as a quick, simple and non-invasive test to stratify SS patients with autoimmune epithelitis, who will potentially be the best candidates for therapies targeting T – B interactions in the context of dysregulated GC responses.

Concluding Remarks

Biomedical discovery is classically based on unifying principles of broad applicability. Similarly, medical practice has traditionally been based on repeated observations and definitions of similarities between patients, that guide treatment decisions on the basis of prior experience. It is increasingly clear, however, that the full realization of the promise of genetic and genomic discovery will require a paradigm shift embracing complexity, heterogeneity, and information-rich decision matrices personalized to individual patients[506,507]. Central to this new era of precision medicine will be the development of drugs to attain control of inflammation and prevention of structural tissue damage. For all autoimmune diseases, future goals involve earlier intervention, induction of immune tolerance, and possibly normalization of immune responses. Critical to this progress will be targeting pathways that maintain disease chronicity but are not involved in host defense against infection.

By providing a better understanding of human Tfr cell biology we further support the importance of the immune system on human pathology both from a fundamental and a clinical perspective.

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ANNEXES

List of Publications Included in the Thesis

Original Research Papers

1. Fonseca VR, Água-Doce A, Maceiras AR, et al. Human blood Tfr cells are indicators of ongoing humoral activity not fully licensed with suppressive function. **Sci Immunol** 2017; 2: eaan1487.
2. Fonseca VR, Romão VC, Água-Doce A, et al. Blood Tfr/Tfh ratio marks ectopic lymphoid structure formation and PD-$1^{+}$$ICOS^{+}$ Tfh cells indicate disease activity in primary Sjögren's syndrome. **Arthritis Rheumatol** 2018 (*in press*).

Invited Reviews

1. Maceiras AR*, Fonseca VR*, Água-Doce A, Graca L. T follicular regulatory cells in mice and men. **Immunology** 2017; 152: 25-35 (*first co-authors).

AUTOIMMUNITY

Human blood T_{fr} cells are indicators of ongoing humoral activity not fully licensed with suppressive function

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Germinal center (GC) responses are controlled by T follicular helper (T_{fh}) and T follicular regulatory (T_{fr}) cells and are crucial for the generation of high-affinity antibodies. Although the biology of human circulating and tissue T_{fh} cells has been established, the relationship between blood and tissue T_{fr} cells defined as $CXCR5^+Foxp3^+$ T cells remains elusive. We found that blood T_{fr} cells are increased in Sjögren syndrome, an autoimmune disease with ongoing GC reactions, especially in patients with high autoantibody titers, as well as in healthy individuals upon influenza vaccination. Although blood T_{fr} cells correlated with humoral responses, they lack full B cell–suppressive capacity, despite being able to suppress T cell proliferation. Blood T_{fr} cells have a naïve-like phenotype, although they are absent from human thymus or cord blood. We found that these cells were generated in peripheral lymphoid tissues before T-B interaction, as they are maintained in B cell–deficient patients. Therefore, blood $CXCR5^+Foxp3^+$ T cells in human pathology indicate ongoing humoral activity but are not fully competent circulating T_{fr} cells.

INTRODUCTION

Germinal center (GC) responses are crucial for the generation of high-affinity antibodies during T-dependent immune responses. Within the GC resides a specialized subset of $CD4^+$ T cells—the T follicular helper (T_{fh}) cells—which are essential for GC development and function (*1*, *2*). It is now clear that T_{fh} cells play a central role in productive vaccine responses, whereas defects in their formation or function can contribute to immunodeficiency or autoimmunity (*3*, *4*). More recently, the discovery of T follicular regulatory (T_{fr}) cells, a subset of suppressive regulatory T (T_{reg}) cells that participate in the GC, added an additional layer of complexity in the biology of GC responses (*5*–*8*).

T_{fr} cells, generally defined by $Bcl\text{-}6^+CXCR5^+PD\text{-}1^+ICOS^+Foxp3^+$, are a distinct subset of thymic $Foxp3^+$ T_{reg} cells present in lymphoid tissues. Like the T_{fh} cell differentiation pathway, T_{fr} cell commitment requires both dendritic cell and B cell interactions, as well as CD28, SAP (SLAM-associated protein), ICOS (inducible costimulator), and PD-1 (programmed cell death–1) signaling (*6*, *9*, *10*). A tight balance between expression of transcription factors Bcl-6 and Blimp-1 regulates the differentiation of T_{fr} cells (*6*). T_{fr} cells have specialized functions in controlling the magnitude of GC responses and in limiting the outgrowth of non–antigen-specific B cell clones (*5*, *6*). However, the precise mechanisms of T_{fr} cell suppression remain elusive, although cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and regulation of metabolic pathways seem to play a key role (*11*–*13*).

Although T_{fh} and T_{fr} cells are characterized by their location in lymphoid tissues, an increasing number of studies have described putative circulating counterparts of these cells in peripheral blood. This is particularly relevant for studying the biology of these cells in humans, because access to secondary lymphoid tissues can be limiting. Human blood $CXCR5^+$ T cells have been established as memory T_{fh}–like cells based on their ability to recapitulate bona fide T_{fh} cell functions: Human blood $CXCR5^+$ T cells can promote plasmablast differentiation, activation-induced cytidine deaminase expression, and class switch recombination by naïve B cells. However, they are phenotypically distinct from tissue T_{fh} cells and do not express the transcriptional repressor Bcl-6 (*14*–*16*). Furthermore, an immunization leading to GC and antibody responses correlates with an increase in the frequency of circulating $ICOS^+$ T_{fh} cells, suggesting that they indicate ongoing T_{fh} cell responses in secondary lymphoid tissues (*14*, *16*–*19*). Human circulating T_{fh} cells comprise a heterogeneous population concerning their phenotype and the quality of help they provide to B cells (*14*, *17*). In mice, $CXCR5^+Foxp3^+$ T_{fr}-like cells were found in peripheral blood after immunization and were shown to represent a circulating counterpart of tissue T_{fr} cells (*9*, *10*).

Although CXCR5-expressing T_{reg} cells and GC Foxp3-expressing T cells have been found in humans (*7*, *20*, *21*), so far, no studies have addressed the biological importance of these putative circulating T_{fr}-like cells in humans. Human tonsil $CD25^+CD69^-$ T cells have been shown to directly suppress B cell responses, but the relationship of these putative T_{reg} cells to $Bcl\text{-}6^+CXCR5^+PD\text{-}1^+ICOS^+Foxp3^+$ T_{fr} cells is unclear (*22*, *23*). Peripheral blood $CXCR5^+$ T_{reg} cells are being studied as circulating T_{fr} cells in many different human diseases, despite the fact that the biological relevance of these cells is unclear (*24*–*28*). Additionally, Foxp3 up-regulation by nonregulatory human T cells and transient CXCR5 expression by T cells undergoing activation challenge the assumption that peripheral blood $CXCR5^+$ T_{reg} cells are bona fide circulating T_{fr} cells (*29*, *30*).

Here, we found that human blood T_{fr} cells, defined as $CXCR5^+Foxp3^+$ T cells, are generated in peripheral lymphoid tissues as humoral immune responses are established. However, in contrast to tissue T_{fr} cells and conventional $CXCR5^-$ T_{reg} cells, circulating T_{fr} cells have a naïve-like phenotype. Our data demonstrate that blood T_{fr} cells are generated following the initial steps that lead to GC responses being distinct from tissue T_{fr} cells.

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RESULTS

Blood T_{fr} cells indicate ongoing GC responses

To address the impact of T_{fr}/T_{fh} ratio in human autoimmunity, we studied Sjögren syndrome (SS), a systemic autoimmune disease characterized by the lymphocytic infiltration of salivary and lachrymal glands, with the formation of ectopic lymphoid structures demonstrating the pathogenic involvement of T-B cell interactions (*31*, *32*). We studied a cohort of 25 patients with recently diagnosed SS according to American-European Consensus Group (AECG) criteria (*33*) under no immunosuppressive treatment other than prednisolone (less than 7.5 mg/day or equivalent) or hydroxychloroquine (table S1). Unexpectedly, we found a notable increased frequency of circulating T_{fr} cells in SS as compared with age-matched healthy donors (HDs) (Fig. 1A and fig. S1, A and B). Whereas SS patients showed fewer T_{reg} and T_{fh} cells per milliliter of blood, in agreement with their lymphopenic state (fig. S1C), the absolute number of $CXCR5^+$ T_{fr} cells was not different when compared with HDs (fig. S1B), in line with the increased frequency of blood $CXCR5^+$ T_{fr} cells. The increased frequency of the $CXCR5^+$ T_{reg} cell subset was specifically increased, providing an explanation for the high T_{reg} cell frequency observed in SS patients (Fig. 1A). SS patients showed a substantial increase in the T_{fr}/T_{fh} ratio compared with HDs (Fig. 1B) (*8*, *10*). Furthermore, among SS patients, the increased T_{fr}/T_{fh} ratio is associated with patients with serum autoantibodies (Fig. 1C). On the contrary, we found no correlation between high T_{fr}/T_{fh} ratio with C-reactive protein and disease activity score [EULAR Sjögren's syndrome disease activity index (ESSDAI)] (Fig. 1, D and E).

Blood and tissue T_{fr} cells present different follicular and regulatory markers

To test whether $CXCR5^+Foxp3^+$ T_{fr} cells in human peripheral blood are circulating counterparts of tissue T_{fr} cells, we studied peripheral blood from a cohort of 42 healthy volunteers between 22 and 92 years (mean age, 46.76 ± 18.14 years; 30 females and 12 males). We found that CXCR5 was expressed by 18.57 ± 6.55% of total T_{reg} cells (defined as $CD4^+CD25^+Foxp3^+$ T cells) (Fig. 2A). The frequency and number of $CXCR5^+Foxp3^+$ T cells did not change with aging (Fig. 2B and fig. S2A).

Because CXCR5 is used to identify human circulating T_{fh} cells, we compared the phenotype of circulating $CXCR5^+Foxp3^+$ T_{fr} cells with that of circulating T_{fh} cells and $CXCR5^-$ conventional T_{reg} cells. Peripheral blood $CXCR5^+Foxp3^+$ T cells share characteristics with both circulating T_{fh} cells and $CXCR5^-$ T_{reg} cells (Fig. 2C). Taking advantage of routine tonsillectomies performed because of tonsil hypertrophy in otherwise healthy children, we compared the cell phenotype of paired blood and

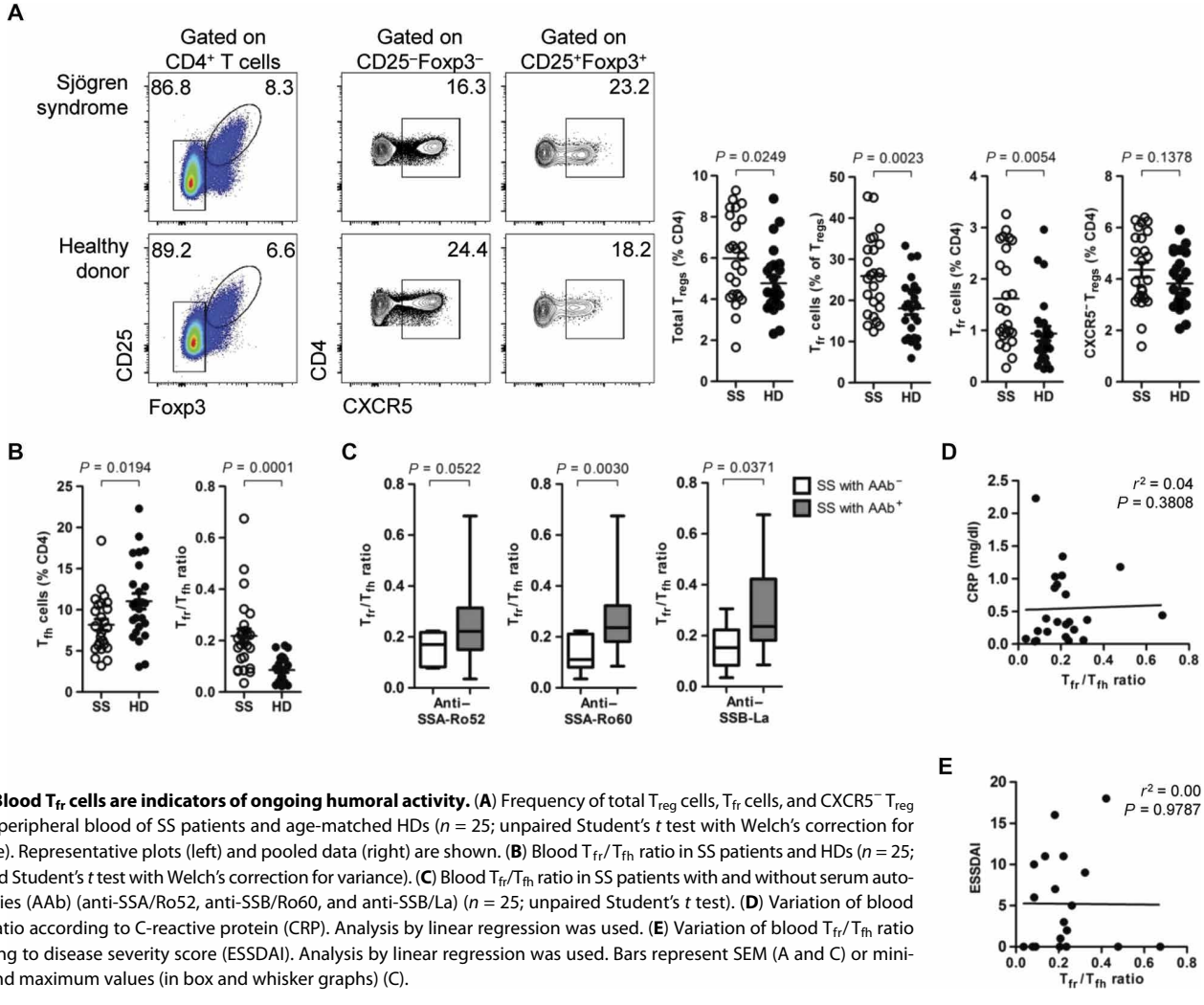

Fig. 1. Blood T_{fr} cells are indicators of ongoing humoral activity. (**A**) Frequency of total T_{reg} cells, T_{fr} cells, and $CXCR5^-$ T_{reg} cells in peripheral blood of SS patients and age-matched HDs ($n = 25$; unpaired Student's t test with Welch's correction for variance). Representative plots (left) and pooled data (right) are shown. (**B**) Blood T_{fr}/T_{fh} ratio in SS patients and HDs ($n = 25$; unpaired Student's t test with Welch's correction for variance). (**C**) Blood T_{fr}/T_{fh} ratio in SS patients with and without serum autoantibodies (AAb) (anti-SSA/Ro52, anti-SSB/Ro60, and anti-SSB/La) ($n = 25$; unpaired Student's t test). (**D**) Variation of blood T_{fr}/T_{fh} ratio according to C-reactive protein (CRP). Analysis by linear regression was used. (**E**) Variation of blood T_{fr}/T_{fh} ratio according to disease severity score (ESSDAI). Analysis by linear regression was used. Bars represent SEM (A and C) or minimum and maximum values (in box and whisker graphs) (C).

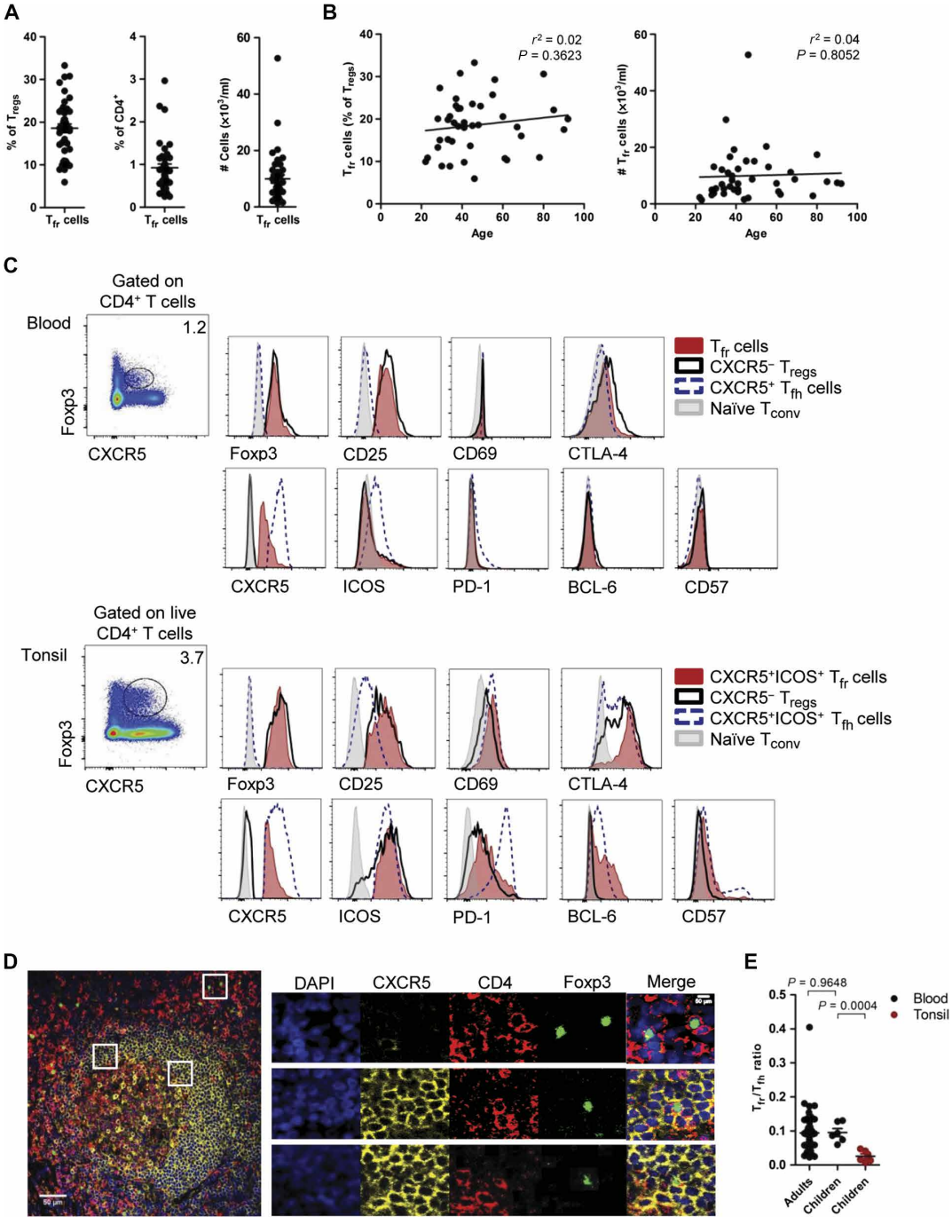

Fig. 2. Blood T_{fr} cells show expression of follicular and regulatory markers. (**A**) $CXCR5^+$ T_{fr} cells constitute 18.57 ± 6.55% of T_{reg} cells (left) and 0.93 ± 0.56% of total $CD4^+$ T cells (middle), representing 9985 ± 9043 cells per milliliter of blood (right) (n = 42; adult HDs). (**B**) Variation of blood T_{fr} cell frequency (left) and absolute number per milliliters of blood (right) according to age (age range, 22 to 92 years) (n = 42; linear regression). (**C**) Expression of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1, Bcl-6, and CD57 by T_{fh} cells (blue), $CXCR5^-$ T_{reg} cells (black), and T_{fr} cells (red) in children blood (top) and tonsils (bottom). Naïve $CD4^+$ T cells were used as control (gray). Representative plots from six healthy children are shown. $CXCR5^+$ subsets in tonsils were defined as $CXCR5^+ICOS^+$ cells (fig. S2B). T_{conv}, conventional T cells. (**D**) Immunofluorescence microscopy of formalin-fixed, paraffin-embedded human tonsils stained for DAPI (blue), CXCR5 (yellow), CD4 (red), and Foxp3 (green). Top, middle, and bottom outlined areas indicate top, middle, and bottom enlarged areas on the right, respectively. Data are representative of tonsil sections from five healthy children. (**E**) Blood T_{fr}/T_{fh} ratio in adult blood, children blood (tonsil donors), and tissues (tonsils). Black and red dots represent blood and tonsil results, respectively (n = 42 for adults and n = 6 for children; Student's t test). Error bars represent SEM.

tissue samples from the same child (Fig. 2C and fig. S2B). We found that circulating T_{fh} cells were phenotypically distinct from their tissue counterparts, in line with previous reports, especially regarding their PD-1, ICOS, and Bcl-6 expression (Fig. 2C) (*14*, *15*). In a similar way, circulating $CXCR5^+Foxp3^+$ T cells were also $ICOS^-PD\text{-}1^-Bcl\text{-}6^-CD57^-$ and consequently distinct from tonsil T_{fr} cells (Fig. 2C). In addition, we confirmed that these cell populations displayed a similar phenotype in adults (fig. S2, C and D). Our results are consistent with murine studies showing that blood and tissue T_{fr} cells are phenotypically distinct (*9*). ICOS was not differentially expressed by T_{reg} and T_{fr} cells in tonsils (Fig. 2C). Bcl-6 expression was not detected in any population by real-time polymerase chain reaction (PCR) (fig. S1F), consistent with previous reports showing that blood T_{fh} cells do not express Bcl-6 (*15*–*18*, *34*). We also confirmed that tissue $CXCR5^+Foxp3^+$ T cells are localized within GCs, therefore corresponding to T_{fr} cells (Fig. 2D and fig. S2G). Curiously, we observed different T_{fr}/T_{fh} ratios in the blood and tonsils (Fig. 2E).

$CXCR5^+Foxp3^+$ T_{fr} cells are a distinct subset of suppressive $Foxp3^+$ T cells

It has been described that CXCR5 expression can transiently occur upon human T cell activation (*30*, *35*, *36*). Moreover, human T cells can also transiently express Foxp3 upon in vitro T cell receptor (TCR) stimulation in a transforming growth factor–β (TGF-β)–dependent manner (*29*, *37*).

To address whether ex vivo CXCR5$^+$Foxp3$^+$ T_{fr} cells were bona fide regulatory cells, we sorted that cell population, as well as CXCR5$^-$ conventional T_{reg} cells (fig. S3, A and B), and cultured them with CellTrace Violet (CTV)–labeled conventional T cells. Proliferation of responder cells was analyzed after 5 days of soluble αCD3 stimulation (Fig. 3A). Blood CXCR5$^+$Foxp3$^+$ T_{fr} cells reduced conventional T cell proliferation (Fig. 3, B and C), definitely demonstrating their regulatory function.

Stability of Foxp3 expression is required for the suppressive function of T_{reg} cells (*38*). To determine whether blood T_{fr} cells have stable

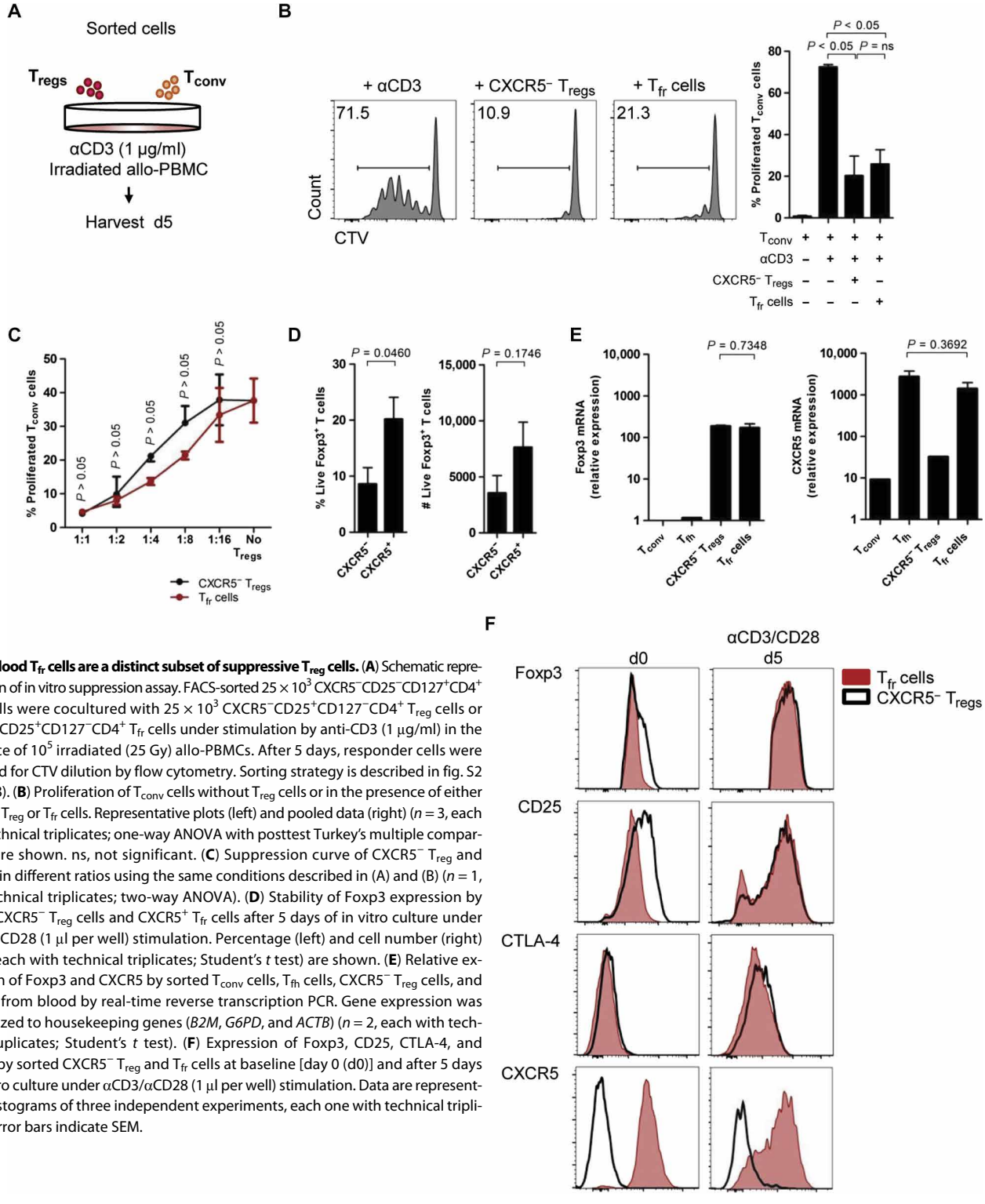

Fig. 3. Blood T_{fr} cells are a distinct subset of suppressive T_{reg} cells. (**A**) Schematic representation of in vitro suppression assay. FACS-sorted 25 × 10^3 CXCR5$^-$CD25$^-$CD127$^+$CD4$^+$ T_{conv} cells were cocultured with 25 × 10^3 CXCR5$^-$CD25$^+$CD127$^-$CD4$^+$ T_{reg} cells or CXCR5$^+$CD25$^+$CD127$^-$CD4$^+$ T_{fr} cells under stimulation by anti-CD3 (1 μg/ml) in the presence of 10^5 irradiated (25 Gy) allo-PBMCs. After 5 days, responder cells were analyzed for CTV dilution by flow cytometry. Sorting strategy is described in fig. S2 (A and B). (**B**) Proliferation of T_{conv} cells without T_{reg} cells or in the presence of either CXCR5$^-$ T_{reg} or T_{fr} cells. Representative plots (left) and pooled data (right) (n = 3, each with technical triplicates; one-way ANOVA with posttest Turkey's multiple comparisons) are shown. ns, not significant. (**C**) Suppression curve of CXCR5$^-$ T_{reg} and T_{fr} cells in different ratios using the same conditions described in (A) and (B) (n = 1, with technical triplicates; two-way ANOVA). (**D**) Stability of Foxp3 expression by sorted CXCR5$^-$ T_{reg} cells and CXCR5$^+$ T_{fr} cells after 5 days of in vitro culture under αCD3/αCD28 (1 μl per well) stimulation. Percentage (left) and cell number (right) (n = 5, each with technical triplicates; Student's t test) are shown. (**E**) Relative expression of Foxp3 and CXCR5 by sorted T_{conv} cells, T_{fh} cells, CXCR5$^-$ T_{reg} cells, and T_{fr} cells from blood by real-time reverse transcription PCR. Gene expression was normalized to housekeeping genes (*B2M*, *G6PD*, and *ACTB*) (n = 2, each with technical duplicates; Student's t test). (**F**) Expression of Foxp3, CD25, CTLA-4, and CXCR5 by sorted CXCR5$^-$ T_{reg} and T_{fr} cells at baseline [day 0 (d0)] and after 5 days of in vitro culture under αCD3/αCD28 (1 μl per well) stimulation. Data are representative histograms of three independent experiments, each one with technical triplicates. Error bars indicate SEM.

Foxp3 expression, we stimulated sorted T_{fr} cells and CXCR5$^-$ T_{reg} cells with anti-CD3/CD28 microbeads for 5 days in the absence of exogenous interleukin-2 (IL-2). In the absence of IL-2, T_{reg} cells do not survive well in culture. Under these conditions, both CXCR5$^-$ T_{reg} cells and T_{fr} cells retain a similar frequency of Foxp3$^+$ cells, albeit lower than in the beginning of the culture (Fig. 3D). The frequency of recovered live Foxp3-expressing cells was slightly higher for sorted T_{fr} cells as compared with CXCR5$^-$ conventional T_{reg} cells. Next, we analyzed the relative expression of Foxp3 and CXCR5 in sorted conventional T cells, T_{fh} cells, T_{fr} cells, and CXCR5$^-$ T_{reg} cells from human blood by real-time PCR. Although Foxp3 protein expression was lower in T_{fr} cells than in CXCR5$^-$ T_{reg} cells (Fig. 2C and fig. S2D), Foxp3 gene expression was similar between the two subsets (Fig. 3E). In addition, circulating T_{fh} and T_{fr} cells also showed comparable CXCR5 gene expression (Fig. 3E).

To investigate whether activation of blood T_{fr} cells triggers up-regulation of Foxp3, CD25, and CTLA-4, a phenomenon known to be associated with increased T_{reg} cell–suppressive function (*38*), we analyzed the phenotype of sorted CXCR5$^+$ and CXCR5$^-$ T_{reg} cells after 5 days of culture in the presence of αCD3/CD28 microbeads. We found an up-regulation of Foxp3 and CD25 by T_{fr} cells, whereas CTLA-4 was increased in both populations (Fig. 3F and fig. S3C). The levels of expression of these markers by blood T_{fr} cells after activation resembled those from tissue T_{fr} cells (compare with Fig. 2C). CXCR5 up-regulation was not detected in sorted CXCR5$^-$ T_{reg} cells, showing that CXCR5$^+$ T_{fr} cells are a distinct subset of human blood T_{reg} cells.

Blood T_{fr} cells do not preferentially suppress humoral responses

To address the function of blood T_{fr} cells, we first investigated whether this population could directly suppress T_{fh} cells. Using a similar in vitro assay used to prove the regulatory capacity of blood T_{fr} cells, but with sorted T_{fh} cells as responders, we found that blood T_{fr} cells strongly suppressed T_{fh} cell proliferation, but without a specific advantage when compared with CXCR5$^-$ T_{reg} cells (Fig. 4A).

Next, to directly assess the impact of blood T_{fr} cells on B cell activation, we used in vitro T-B cocultures in the presence of staphylococcal enterotoxin B (SEB) superantigen (Fig. 4C). After 5 days of culture, B cells up-regulated CD38 and down-regulated immunoglobulin D (IgD) only in the presence of T_{fh} cells (Fig. 4C). Both CXCR5$^-$ and CXCR5$^+$ T_{reg} cells impaired the generation of CD38$^+$IgD$^-$ GC-like B cells. Consistent with our results from suppression assays with T_{fh} cells (Fig. 4A), T_{fh} cell proliferation was similarly inhibited by CXCR5$^-$ and CXCR5$^+$ T_{reg} cells (Fig. 4, C and D, and fig. S4A). As expected, T_{fh} cells showed better proliferation responses in coculture with B cells.

To further address the function of blood T_{fr} cells on humoral responses, we analyzed class switch recombination by naïve B cells 10 days after superantigen stimulation. We found that blood T_{fr} cells, although able to reduce activation of naïve B cells and proliferation of T_{fh} cells as shown before, did not limit class switch recombination by B cells because no impact on IgA or IgG production was observed (Fig. 4E). On the contrary, CXCR5$^-$ T_{reg} cells efficiently suppressed humoral responses (Fig. 4E).

CXCR5/CXCL13-dependent migration to GC is critical for suppression of humoral responses by T_{fr} cells (*5*, *7*), and plasma CXCL13 levels have been correlated to ongoing GC responses in humans (*39*). To prove that blood T_{fr} cells were capable of migrating toward a CXCL13 gradient, we conducted in vitro chemotaxis assays with sorted populations from human peripheral blood. We found that, although the CXCR5 mean fluorescence intensity (MFI) of peripheral T_{fh} and T_{fr} cells was slightly different (Fig. 2C and fig. S2D), both populations shared their ability to migrate toward a CXCL13 gradient, showing functional capacity of blood T_{fr} cells to enter CXCL13-enriched tissues (Fig. 4F).

Blood T_{fr} cells have a distinctive naïve-like phenotype

To explain the unexpected observation that blood T_{fr} cells do not suppress antibody production, we hypothesized that this population could represent thymus-derived precursors of T_{fr} cells not yet fully committed to regulate humoral responses. We found that blood T_{fr} cells were predominantly CD45RO$^-$Foxp3^{lo} resting T_{reg} cells (Fig. 5A), expressing high levels of CD45RA, CCR7, CD62L, and CD27 and low levels of human leukocyte antigen–DR (HLA-DR), reminiscent of a naïve phenotype (Fig. 5B). Virtually, all blood T_{fr} cells were quiescent Ki-67$^-$ nonproliferating cells when analyzed ex vivo (Fig. 5C). Moreover, circulating T_{fr} cells were virtually devoid of CD45RO$^+$CCR7$^-$ effector memory cells, in notable contrast to CXCR5$^-$ T_{reg} cells, a phenotype more similar to circulating T_{fh} cells (Fig. 5D). Although the vast majority of blood T_{fh} cells were CD45RO$^+$CCR7$^+$ central memory cells, consistent with previous reports (*14*–*17*), a substantial proportion of T_{fr} cells were CD45RO$^-$CCR7$^+$ naïve cells (Fig. 5D). Furthermore, the few CD45RO$^-$ T_{fh} cells did not express high levels of CD45RA, indicating that those cells were not really naïve, in contrast to T_{fr} cells (fig. S5A). Therefore, blood T_{fr} cells constitute a pool of naïve resting cells.

To test whether blood T_{fr} cells were thymus-derived precursors of tissue T_{fr} cells, we analyzed the frequency of these cells according to age. Contrary to thymus-derived naïve T_{reg} cells, CD45RO$^-$CCR7$^+$ naïve T_{fr} cells did not decrease with increasing age (Fig. 5E). In addition, the expression of CD31, a marker used to identify recent thymic emigrants in human blood (*40*–*42*), was not specifically enriched in the population (fig. S5, B and C). Although these observations suggest that blood T_{fr} cells are not a thymic population, this was not conclusive. Therefore, we directly examined CXCR5-expressing T cells in the human thymus and neonatal cord blood. There was not a population of CXCR5$^+$ T_{reg} cells detected in any of those tissues (Fig. 5, F and G, and fig. S5D). Although CXCR5-expressing T_{reg} cells were not found in cord blood, some Foxp3$^+$ cells expressed CD45RO, suggesting that additional activation signals not present before birth are required to shape a CXCR5 phenotype in circulating T_{reg} cells. Consistent with our previous data, ICOS$^+$ T_{reg} cells were detected in cord blood, indicating that ICOS cannot be used as a specific follicular marker in circulating human T_{reg} cells (Fig. 5H).

Blood T_{fr} cells emerge from lymphoid organs before B cell interaction

Having demonstrated that circulating T_{fr} cells did not egress from the thymus, we investigated whether T_{fr} cells recirculate from secondary lymphoid tissues before being fully committed to tissue T_{fr} cells. We compared both CXCR5$^+$ and CXCR5$^-$ T_{reg} cell subsets from children paired blood and tissue (tonsils) concerning their effector memory, central memory, and naïve composition. We found that CD45RO$^+$CCR7$^-$ effector T_{fr} cells were present in lymphoid tissues but not in the blood, suggesting that effector T_{fr} cells are selectively retained in tissues, similarly to effector T_{fh} cells (Fig. 6A). Therefore, it is unlikely that blood CD45RO$^-$ T_{fr} cells derive from the fully mature tissue T_{fr} cells that express CD45RO, as the few CD45RA

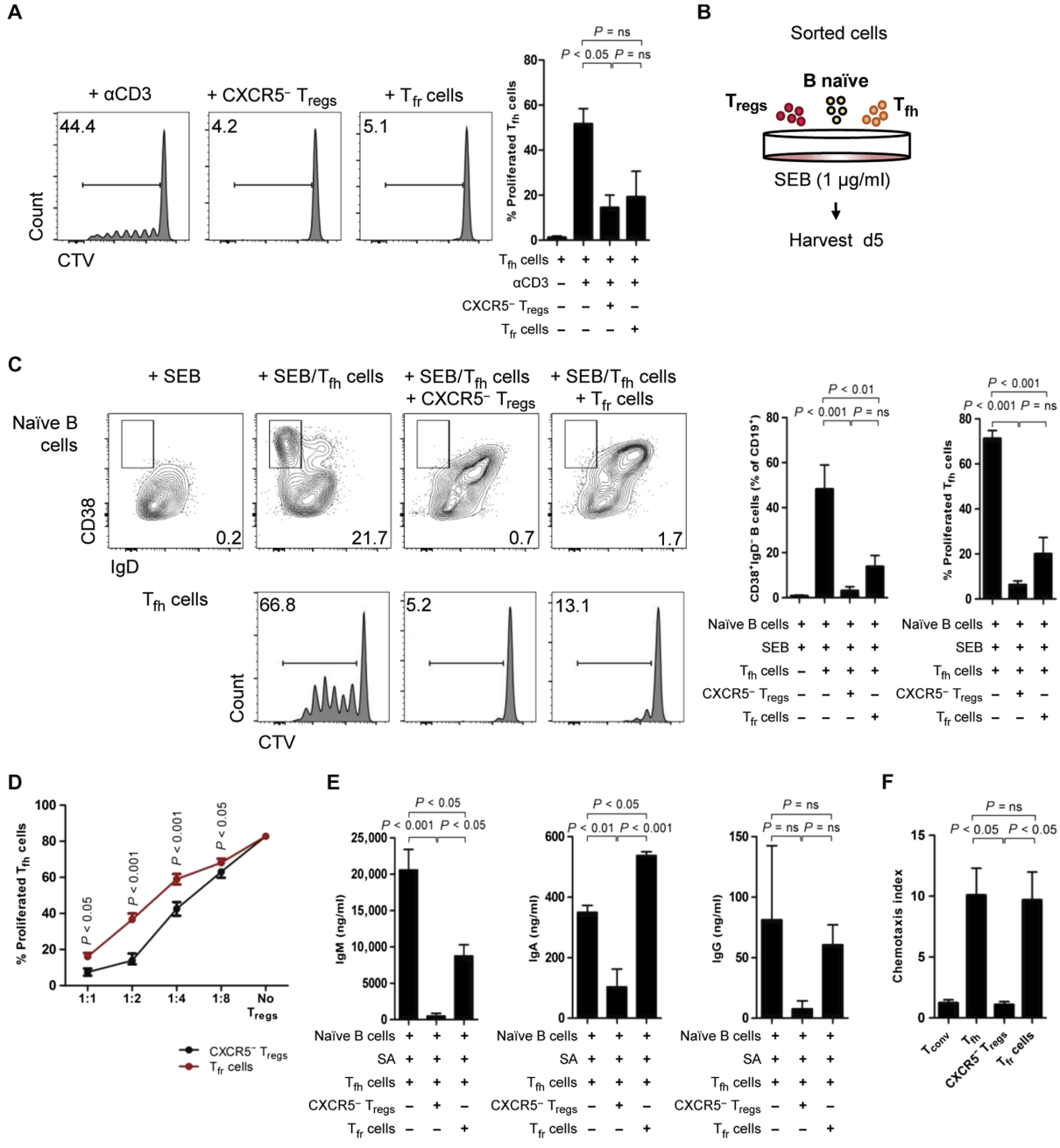

Fig. 4. Blood T_{fr} cells do not show specialized humoral regulatory capacity. (**A**) Proliferation of $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells without T_{reg} cells or in the presence of either $CXCR5^-$ T_{reg} or T_{fr} cells after 5 days of in vitro culture as described in Fig.3A. Representative plots (left) and pooled data (right) ($n = 3$, each with technical triplicates; one-way ANOVA with posttest Turkey's multiple comparison) are shown. (**B**) Schematic representation of suppression coculture assay. FACS-sorted 25×10^3 $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells (or $CXCR5^-CD25^-CD127^+CD4^+$ T_{conv} cells) were cocultured for 5 days with 25×10^3 $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells (or $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells) under stimulation by SEB (1 μg/ml) and in the presence of 30×10^3 $CD27^-IgD^+CD19^+$ naïve B cells. (**C**) Up-regulation of CD38 and down-regulation of IgD by naïve B cells (top) and proliferation of T_{fh} cells by CTV dilution (bottom) without T_{reg} cells or in the presence of either $CXCR5^-$ T_{reg} or T_{fr} cells. Representative plots (left) and pooled data (right) ($n = 5$, each with technical triplicates; one-way ANOVA with posttest Turkey's multiple comparisons) are shown. (**D**) Suppression curve of $CXCR5^-$ T_{reg} and T_{fr} cells in different ratios using the same conditions described in (B) and (C) ($n = 1$, with technical triplicates; two-way ANOVA). (**E**) ELISA determination of IgA, IgM, and total IgG in supernatants after 10 days of in vitro coculture performed as described in (D) but using SEB (1 μg/ml) + SEA (10 g/ml) + SEE (10 ng/ml) + TSST-1 (10 ng/ml) as superantigen stimulation ($n = 3$, each with technical triplicates; one-way ANOVA with posttest Turkey's multiple comparisons). (**F**) In vitro migration of 75×10^3 sorted T_{conv} cells, T_{fh} cells, $CXCR5^-$ T_{reg} cells, and T_{fr} cells toward a CXCL13 gradient (2 μ/ml) expressed by chemotaxis index ($n = 3$, each with technical triplicates; one-way ANOVA with posttest Turkey's multiple comparisons). Error bars indicate SEM. SA, superantigens.

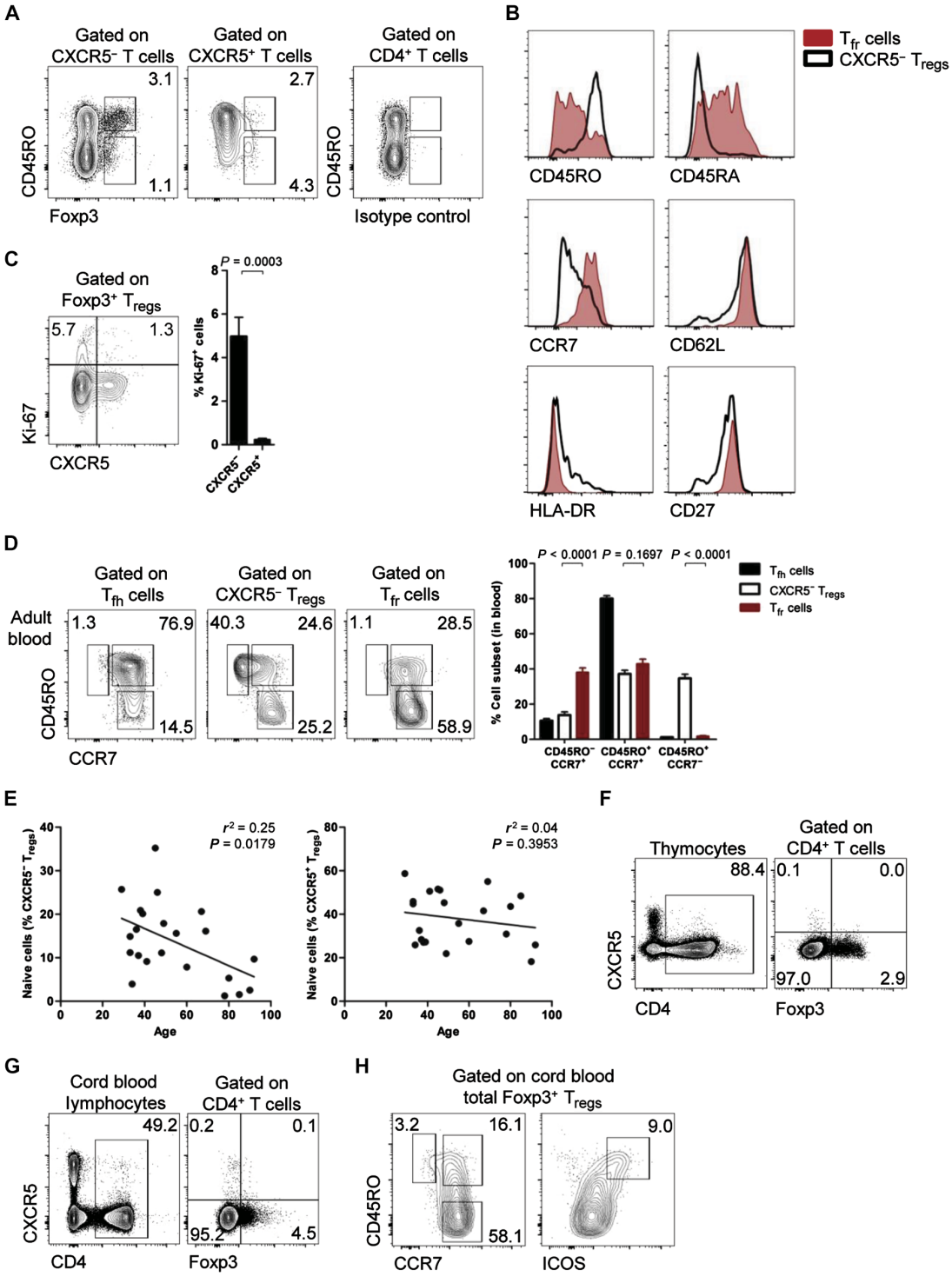

Fig. 5. Blood T_{fr} cells are immature but are not committed in the thymus. (**A**) Backgate of $CXCR5^-$ and $CXCR5^+$ T_{reg} cells according to CD45RO and Foxp3 expression. (**B**) Expression of CD45RO, CD45RA, CCR7, CD62L, HLA-DR, and CD27 by T_{fr} cells (red) and $CXCR5^-$ T_{reg} cells (black) in the blood. (**C**) Expression of Ki-67 by $CXCR5^+$ T_{reg} cells and $CXCR5^-$ T_{reg} cells in the blood ($n = 22$; Student's t test). (**D**) $CD45RO^+CCR7^-$ effector memory, $CD45RO^+CCR7^+$ central memory, and $CD45RO^-CCR7^+$ naïve subsets of T_{fr} cells and $CXCR5^-$ T_{reg} cells in adult blood. Representative plots (left) and pooled data (right) ($n = 22$; Student's t test). T_{fh} cells are represented in blue, $CXCR5^-$ T_{reg} cells in black, and T_{fr} cells in red. (**E**) Variation of $CD45RO^-CCR7^+$ naïve T_{fr} cell and $CXCR5^-$ T_{reg} cell frequency in blood according to age ($n = 22$; linear regression). (**F**) Expression of CXCR5 by $Foxp3^+CD4^+$ thymocytes ($n = 4$). (**G**) Expression of CXCR5 by cord blood $Foxp3^+CD4^+$ T cells ($n = 3$). (**H**) Expression of CD45RO, CCR7, and ICOS by cord blood T_{reg} cells ($n = 3$). Bars represent SEM.

reexpressing end-stage memory $CD4^+$ T cells do not become $CD45RO^-$ (fig. S6A) (*43*, *44*).

Our data suggest that blood T_{fr} cells are generated in secondary lymphoid tissue before full differentiation toward mature T_{fr} cells. It has been known that full differentiation of follicular T cells requires a two-step process, with an initial activation mediated by dendritic cells and a subsequent B cell interaction in the T-B border. We investigated whether blood T_{fr} cells, given their immature phenotype, could be generated before the B cell interactions required for acquisition of terminal differentiation. To investigate this issue, we analyzed peripheral blood from X-linked agammaglobulinemia [Bruton's tyrosine kinase (BTK)–deficient] patients, with a complete absence of $CD19^+$ cells. We observed a notable decrease in blood T_{fh} cells in those patients, in line with previous reports (Fig. 6B) (*45*). However, frequency of blood T_{fr} cells was not decreased in B cell–deficient patients (Fig. 6B). These observations are conclusive in establishing that blood T_{fr} cells enter the circulation before B cell contact, whereas most of the blood T_{fh} cells require B cell interactions. To investigate whether $CD45RO^+$ and $CD45RO^-$ blood T_{fr} cells could discriminate between T_{fr} cells recirculating before and after B cell interaction, we analyzed these two populations in peripheral blood of patients with B cell deficiency, as well as in SS patients. We found no differences in $CD45RO^+$ or $CD45RO^-$ T_{fr} cells in these two diseases (fig. S5E), although CD45RO up-regulation occurs irrespective of B cell interaction on T_{fr} cells.

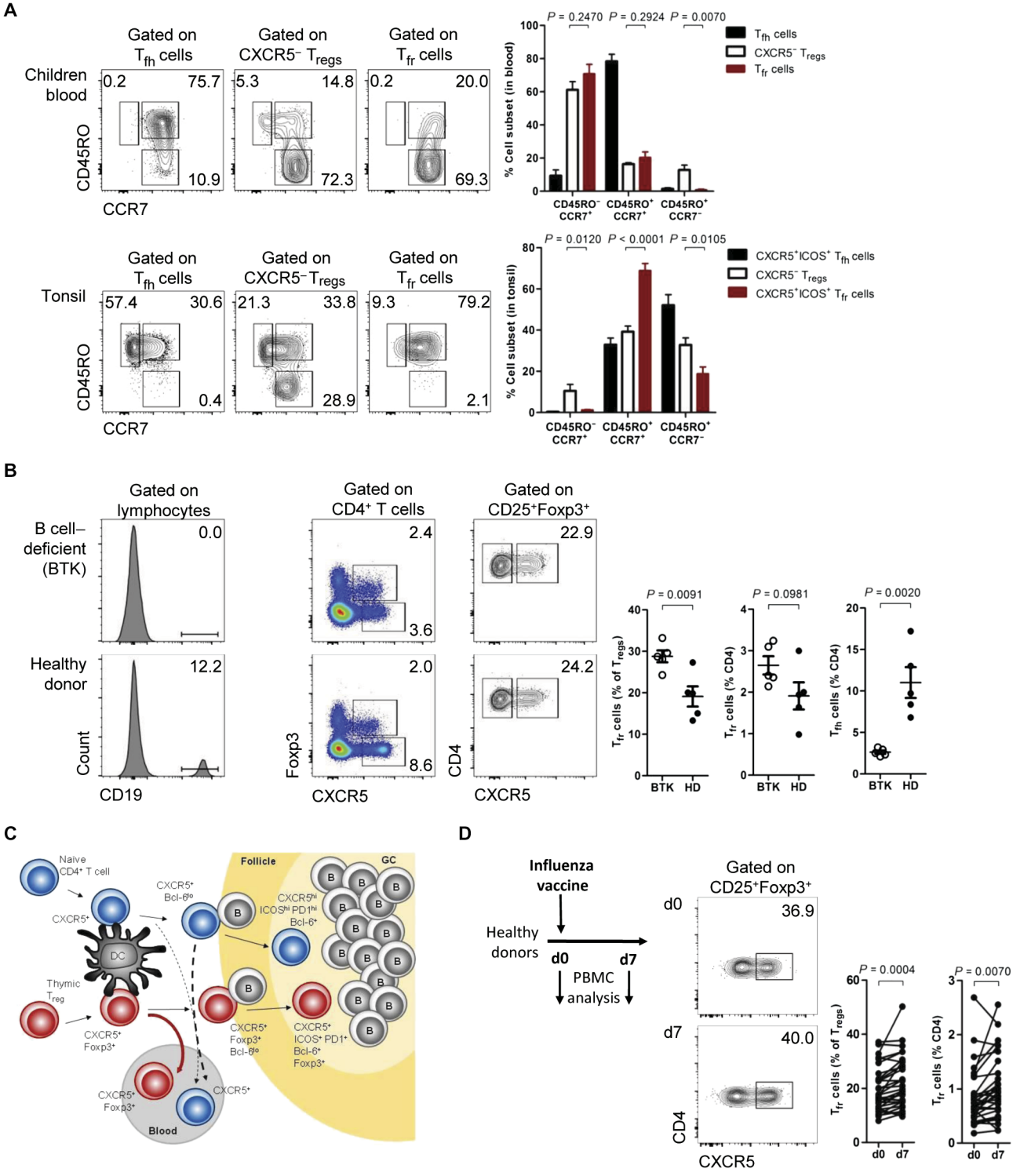

Fig. 6. Blood T_{fr} cells are lymphoid tissue–derived T_{fr} precursors. (**A**) $CD45RO^+CCR7^-$ effector memory, $CD45RO^+CCR7^+$ central memory, and $CD45RO^-CCR7^+$ naïve subsets of T_{fr} cells and $CXCR5^-$ T_{reg} cells in children blood (top) and tissues (bottom). Representative plots (left) and pooled data (right) ($n = 6$; Student's t test) are shown. T_{fh} cells are represented in blue, $CXCR5^-$ T_{reg} cells in black, and T_{fr} cells in red. $CXCR5^+$ subsets in tonsils were defined as $CXCR5^+ICOS^+$ cells (fig. S2B). (**B**) Blood T_{fh} and T_{fr} cells from X-linked agammaglobulinemia (BTK-deficient) patients compared with sex- and age-matched HDs. Representative plots (left) and pooled data (right) ($n = 5$; Student's t test) are shown. (**C**) Model of $CXCR5^+$ T_{fh} and T_{reg} cell generation and recirculation in humans upon antigen stimulation. T_{fh} cells are shown in red, and T_{fr} cells in blue. DC, dendritic cell. (**D**) Frequency of peripheral blood T_{fr} cells on the day of influenza vaccination (d0) and 7 days later in healthy volunteers. Schematic representation and representative plots (left) and pooled data (right) ($n = 32$; Student's t test) are shown. Bars represent SEM.

We therefore hypothesized that blood T_{fr} cells are generated in secondary lymphoid tissue and enter the circulation before full differentiation toward tissue T_{fr} cells (Fig. 6C). To test our model in vivo, we analyzed samples from healthy adults undergoing influenza vaccination. Previous studies have shown a positive correlation between circulating T_{fh} cell subsets and antibody responses after influenza vaccination in healthy adults (*15*, *18*, *19*). We therefore analyzed the impact of vaccination in circulating T_{fr} cells. Consistent with our hypothesis, we found that circulating T_{fr} cells increased on day 7 after influenza vaccination (Fig. 6D). This observation is in line with our prediction that, during ongoing GC responses, T_{fr} cells are generated and some exit from the tissue to the peripheral blood.

DISCUSSION

Our comprehensive evaluation of human T_{fr} supports a model in which blood T_{fr} cells are generated following the initial steps that lead to GC responses in secondary lymphoid tissues, exiting the tissue before interactions with B cells that are required for complete differentiation toward tissue-resident T_{fr} cells. Although some studies have quantified blood $CXCR5^+$ T_{reg} cells as circulating T_{fr} cells in different diseases, the human biology of $CXCR5^+$ T_{reg} cells remains elusive (*24*, *25*, *27*, *46*, *47*). Moreover, most of the literature studies define blood T_{fh} cells as cells that contain both T_{fh} and $CXCR5^+$ T_{fr} cells, whereas many other studies identify T_{reg} cells as a mixture of bona fide conventional T_{reg} cells and $CXCR5^+$ T_{fr} cells. Hence, results may be confounded by combining effector and regulatory cell populations. As an example, our cohort of SS patients shows an increase in the frequency of $Foxp3^+$ T_{reg} cells compared with the control population. However, only $CXCR5^+$ T_{fr} cells, and not conventional $CXCR5^-$ T_{reg} cells, are increased in those patients. As a consequence, the apparent increase of T_{reg} cells in the blood of SS patients is an increase of $CXCR5^+$ T_{fr} cells that reflect the ongoing humoral activity. It was the search for an explanation for this apparent counterintuitive observation that led us to establish the ontogeny and function of human circulating T_{fr} cells.

We found that T_{fr} cells in tonsils have follicular and regulatory markers and were found within GCs, whereas blood T_{fr} cells do not express ICOS, PD-1, or Bcl-6, apparently diverging these cells from follicular imprinting. Previous studies have described low ICOS and PD-1 expression and no Bcl-6 expression in human blood T_{fh} cells (*14*). In mice, blood T_{fr} cells have also lower expression of ICOS (*9*). It was also reported that murine circulating T_{fr} cells can bypass the B cell zone and do not gain full activation as part of a memory programmed state (*9*). In line with these studies, the absence of ICOS, PD-1, and Bcl-6 from human blood $CXCR5^+$ T_{fr} cells does not exclude their follicular ontogeny.

Our results show key differences between mice and humans regarding the function of blood $CXCR5^+$ T_{fr} cells: Although murine blood T_{fr} cells appear to be specialized in suppressing antibody production (despite their lower suppressive capacity when compared with tissue T_{fr} cells) (*9*, *10*, *12*, *13*), human blood T_{fr} cells do not have the ability to fully suppress humoral responses.

Nevertheless, we found that blood T_{fr} cells specifically migrated toward CXCL13 gradient, suggesting that these cells have the capacity to reach the follicles. $CXCR5^-$ conventional T_{reg} cells did not up-regulate CXCR5 upon in vitro activation, further confirming CXCR5-expressing T_{fr} cells as a distinctive subset.

We also found that blood T_{fr} cells have a prominent naïve phenotype. However, they are absent from the thymus and cord blood (where activated T_{reg} cells can already be found). These observations provide compelling evidence that activation signals generated in peripheral lymphoid organs are required to shape a $CXCR5^+$ phenotype on human $Foxp3^+$ T cells. Conversely, tissue T_{fr} cells are almost all $CD45RO^+$ antigen-experienced effector cells. Together, these observations led us to hypothesize that blood T_{fr} cells leave lymphoid tissues as immature cells before B cell interaction in T-B border and full differentiation into T_{fr} cells. This view was supported by the presence of blood T_{fr} cells in peripheral blood of patients lacking B cells due to genetic defects. This finding provides an explanation for the incomplete suppressive function of blood T_{fr} cells.

An important limitation of our study is the difficulty to isolate tissue T_{fr} cells for functional assays because CD25 and CD127 are not reliable to identify tonsil Foxp3-expressing cells. However, the phenotype between blood and tissue T_{fr} cells is remarkably different, in particular, with respect to maturation markers.

Our results from vaccination and SS patient cohorts show that blood T_{fr} cells are indicative of ongoing humoral activity. In SS patients, where ongoing GC reactions promote the production of autoantibodies (*31*, *32*), blood T_{fr} cells were substantially increased (directly contributing to an increased T_{fr}/T_{fh} ratio). Although we expected to find a decrease in this putative humoral suppressive cell population in autoimmune conditions, our results suggest that blood T_{fr} cells indicate ongoing humoral activity and are not a measurement of suppressive potential. This is in line with recently published reports showing an increase in blood $CXCR5^+$ T_{reg} cells in other autoimmune conditions and infectious diseases (*27*, *28*, *47*). Therefore, studies regarding blood $CXCR5^+$ T_{reg} cells in different human settings should be carefully interpreted.

Given that circulating T_{fr} cells have an immature phenotype, it is not unexpected that blood T_{fr} cells are not fully endowed with suppressive function, because the suppressive capacity of conventional T_{reg} cells has been ascribed predominantly to those cells with a more mature phenotype. Although the TCR repertoire of T_{fr} cells is different from T_{fh} and probably skewed toward autoantigens (*48*), it is possible that circulating T_{fr} cells represent a pool of cells ready to be recruited into subsequent GC responses as they retain the ability to migrate toward CXCL13.

In conclusion, our data support a model in which $CXCR5^+Bcl\text{-}6^-$ T cells egress from secondary lymphoid tissues during antigen-driven immune responses. Whereas the frequency of blood T_{fh} cells is reduced in the absence of B cells, T_{fr} cells do not require interactions with B cells. Thus, the acquisition of a $CXCR5^+Foxp3^+$ phenotype in the tissues precedes access to the follicle, where the cells acquire a fully mature phenotype. As a consequence, circulating T_{fr} cells represent lymphoid tissue–derived T_{fr} precursors not yet endowed with full B cell and humoral regulatory function.

MATERIALS AND METHODS

Study design

Sample sizes were estimated on the basis of previous studies and according to each cohort (see the next section). No outliers were excluded. The number of biological and technical replicates is stated in the figure legends. Human samples from different conditions were used (see the next section) with appropriate age-matched controls. This experimental study was performed unblinded.

Human samples

Fresh peripheral blood samples were collected from patients referred to the Rheumatology Department of Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, for salivary gland biopsy due to clinical suspicion of SS. Blood samples were collected on the day of salivary gland biopsy. All patients with exclusion criteria for SS (*33*) or treated with biologic drugs, disease-modifying antirheumatic drugs, or prednisolone (more than 7.5 mg/day) were excluded. Patients diagnosed with an infectious disease in the previous month were also excluded, as well as those who received any vaccine in the same period of time. Patients were diagnosed as having SS if they met the AECG diagnosis criteria ($n = 25$) (*33*). Routine C-reactive protein plasma levels (mg/dl) closest to blood collection were used. Age-matched healthy volunteers (from the cohort described below) were used for statistical comparison. Fresh peripheral blood samples were collected from healthy adult volunteers ($n = 42$). Fresh buffy coats (blood collection in less than 24 hours) were used for in vitro suppression and coculture assays. Tonsils and peripheral blood samples were collected from healthy children submitted to tonsillectomy due to tonsil hypertrophy ($n = 6$). Children with any clinical condition, under any drug treatment, or submitted to tonsillectomy due to chronic tonsillitis were excluded. Umbilical cord blood samples were collected from healthy pregnant women during delivery ($n = 3$). Thymus tissue was collected from children submitted to cardiac surgery due to congenital heart disease who were otherwise healthy ($n = 4$). Blood samples were also collected from X-linked agammaglobulinemia (BTK-deficient) patients during routine blood tests ($n = 5$). All blood samples were collected in EDTA-coated tubes. These studies were approved by the Lisbon Academic Medical Center Ethics Committee (reference no. 505/14). Informed consent was obtained from all adult volunteers, parents, or legal guardians.

For vaccination studies, we used healthy adult volunteers ($n = 32$) recruited from the Cambridge BioResource as part of the vaccination study during the 2014–2015 winter season. Participants were excluded if they have had a previous adverse reaction to any vaccination, have a known allergy to any components of the vaccine, were taking immunomodulating medication, and are pregnant or breastfeeding. Participants were administered the inactivated influenza vaccine (split virion) BP vaccine (Sanofi Pasteur) by intramuscular injection in the right deltoid. Blood samples were collected in EDTA-coated tubes on the day of vaccination (before administration of the vaccine) and 7 days after vaccination. The influenza vaccination study protocol was approved by the Health Research Authority, National Research Ethics Service Committee South Central, Hampshire A, UK (REC reference: 14/SC/1077).

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll gradient medium (Histopaque-1077, Sigma-Aldrich) using SepMate tubes (STEMCELL Technologies). Lymphocytes from tonsils and thymocytes were also isolated by Ficoll gradient medium after mechanical disruption. Before cell sorting, PBMCs from buffy coats were enriched for $CD4^+$ T cells using MojoSort Human CD4 T Cell Isolation Kit (BioLegend). The $CD4^+$ fraction was used for cell sorting of $CD4^+$ T cell subsets. The $CD4^-$ fraction was used for cell sorting of naïve B cells (see fig. S1A for sorting strategy). For flow cytometry, cells were stained with anti–Bcl-6 (K112-91, BD Biosciences), anti-CCR7 (#150503, R&D Systems), anti-CD127 (eBioRDR5, eBioscience), anti-CD19 (HIB19, BioLegend), anti-CD25 (BC96, eBioscience), anti-CD27 (LG.7F9, eBioscience), anti-CD3 (OKT3, eBioscience), anti-CD31 (WM-59, eBioscience), anti-CD38 (HB-7, BioLegend), anti-CD4 (OKT4, BioLegend), anti-CD45RA (HI100, eBioscience), anti-CD45RO (UCHL1, BioLegend), anti-CD57 (HNK-1, BioLegend), anti-CD62L (DREG-56, BioLegend), anti-CD69 (FN30, BioLegend), anti-CD8 (RPA-T8, eBioscience), anti–CTLA-A (L3D10, BioLegend), anti-CXCR5 (J252D4, BioLegend), anti-Foxp3 (PCH101, eBioscience), anti–HLA-DR (G46-6, BD Biosciences), anti-ICOS (C398.4A, BioLegend), anti-IgD (IA6-2, BioLegend), anti–Ki-67 (Ki-67, BioLegend), and anti–PD-1 (EH12.2H7, BioLegend). For Bcl-6, CTLA-4, Foxp3, and Ki-67 intracellular staining, Foxp3 Fix/Perm Kit (eBioscience) was used according to the manufacturer's instructions. For cell viability staining, Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used. CellTrace Violet Cell Proliferation Kit (Life Technologies) was used for cell proliferation assessment. Cell sorting was performed in Aria IIu and Aria III instruments (BD Biosciences). Flow cytometry analysis was performed in an LSRFortessa instrument (BD Biosciences) and further analyzed with FlowJo v10 software (Tree Star).

Cell culture and functional assays

For in vitro suppression assays, 25×10^3 $CXCR5^-CD25^-CD127^+CD4^+$ conventional T cells or 25×10^3 $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells were plated with $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells or $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells in a 1:1 ratio. Cells were cultured with anti-CD3 (1 μg/ml) (OKT3, eBioscience) in the presence of 10^5 irradiated (25 Gy) allo-PBMCs. After 5 days, cells were harvested and responder cells were analyzed for CTV dilution by flow cytometry. For TCR stimulation assays, 25×10^3 $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells and $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells were plated with anti-CD3/anti-CD28 MACSiBead particles (1 μl per well) (T Cell Activation Kit, Miltenyi Biotec). For coculture in vitro suppression assays, 25×10^3 $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells were plated with $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells or $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells in a 1:1 ratio in the presence of 30×10^3 $CD27^-IgD^+CD19^+$ naïve B cells. Cells were cultured with SEB (1 μg/ml) (Sigma-Aldrich). After 5 days, responder T_{fh} cells were analyzed for CTV dilution, B cells for CD38 up-regulation, and T_{reg} cells for follicular and activation markers. For immunoglobulin measurement, 25×10^3 $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells were plated with $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells or $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells in a 1:1 ratio in the presence of 30×10^3 $CD27^-IgD^+CD19^+$ naïve B cells. Cells were cultured with SEB (1 μg/ml) (Sigma-Aldrich) + staphylococcal enterotoxin A (SEA) (10 ng/ml) (Toxin Technology) + staphylococcal enterotoxin E (SEE) (10 ng/ml) (Toxin Technology) + toxic shock syndrome toxin–1 (TSST-1) (10 ng/ml) (Toxin Technology). After 10 days, supernatants were collected and immunoglobulin concentration was determined by enzyme-linked immunosorbent assay (ELISA). Cultures were performed in U-shaped 96-well plates in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 1% Hepes (Sigma-Aldrich), 1% sodium pyruvate (Life Technologies), 1% penicillin-streptomycin (Life Technologies), and 0.05% gentamicin (Life Technologies) in 37°C, 5% CO_2 incubator conditions.

Enzyme-linked immunosorbent assay

IgA, IgM, and total IgG concentrations were determined in supernatants from T-B coculture (as described above) by ELISA using Human ELISA Ready Set Go Kit according to the manufacturer's instructions (eBioscience).

Migration assays

For in vitro chemotaxis assays, 75×10^3 $CXCR5^-CD25^-CD127^+CD4^+$ conventional T cells, $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells, $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells, and $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells were loaded on top wells of HTS Transwell 96-well permeable supports (5-μm pore size) (Corning). Plain RPMI 1640 (Life Technologies) or medium supplemented with CXCL13 (0.2 μg/ml) (PeproTech) was added to the bottom wells of the plate. After 4 hours of incubation (37°C, 5% CO_2), filters were removed and cells that migrated to the lower chamber were counted in an LSRFortessa instrument (BD Biosciences) and further analyzed with FlowJo v10 software (Tree Star). Chemotaxis index was calculated as the ratio of cells migrating toward CXCL13 and cells randomly migrating.

Real-time reverse transcription PCR

Total RNA was extracted and reverse-transcribed from FACS (fluorescence-activated cell sorting)–sorted $CXCR5^-CD25^-CD127^+CD4^+$ conventional T cells, $CXCR5^+CD25^-CD127^+CD4^+$ T_{fh} cells, $CXCR5^-CD25^+CD127^-CD4^+$ T_{reg} cells, and $CXCR5^+CD25^+CD127^-CD4^+$ T_{reg} cells using RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA was generated using SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Real-time PCR was set up with Power SYBR Green PCR Master Mix (Applied Biosystems) and performed on ViiA 7 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The expression of each gene was normalized to housekeeping genes (*B2M*, *ACTB*, or *G6PD*) and calculated by change-in-threshold method (ΔC_T) using QuantStudio Real-Time PCR software v1.1 (Applied Biosystems). The following primers (Invitrogen) were used: Foxp3, 5′-GCAAATGGTGTCTGCAAGTG-3′ (forward) and 5′-GCCCTTCTCATCCAGAAGAT-3′ (reverse); CXCR5, 5′-CTGGAAATGGACCTCGAGAA-3′ (forward) and 5′-GCAGGGCAGAGATGATTTTC-3′ (reverse); Bcl-6, 5′-TTCCGCTACAAGGGCAAC-3′ (forward) and 5′-CGAGTGTGGGTTTTCAGGTT-3′ (reverse); B2M, 5′-TATGCCTGCCGTGTGAACCAT-3′ (forward) and 5′-CGGCATCTTCAAACCTCCATG-3′ (reverse); ACTB, 5′-CTCTTCCAGCCTTCCTTCCT-3′ (forward) and 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse); G6PD, 5′-CCAAGCCCATCCCCTATATT-3′ (forward) and 5′-CCACTTGTAGGTGCCCTCAT-3′ (reverse).

Immunofluorescence microscopy

After paraffin removal and antigen retrieval by heat (HIER pH 9, Leica Biosystems), 3-μm sections of formalin-fixed, paraffin-embedded human tonsil were stained with anti-human CXCR5–Alexa Fluor 488 (J252D4, BioLegend), anti-human CD4 (SP35, Cell Marque), and anti-human Foxp3 (PCH101, eBioscience) primary antibodies. Alexa Fluor 488 (anti-mouse), Alexa Fluor 546 (anti-rabbit), and Alexa Fluor 488 (anti-rat) were used as secondary antibodies. 4′,6-Diamidino-2-phenylindole (DAPI) was used as nuclei counterstaining. Images were acquired with ZEN 2012 software on a Zeiss LSM 710 confocal point-scanning microscope (Carl Zeiss) using a dry Plan-Apochromat 20× objective (×200 magnification) and with a numerical aperture of 0.80. Images were further analyzed using ImageJ Fiji software.

Statistical analysis

Unpaired, paired Student's *t* test, one-way analysis of variance (ANOVA) with posttest Turkey's multiple comparisons, and two-way ANOVA with posttest Bonferroni's multiple comparison were used as described. Linear regression analysis was also conducted for some data. Results are presented as means ± SD. *P* values of less than 0.05 were considered statistically significant. GraphPad Prism v5 software was used for statistical analysis.

SUPPLEMENTARY MATERIALS

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Table S1. Clinical characteristics of 25 patients with primary (pSS) and secondary (sSS) Sjögren syndrome.

Fig. S1. Gating strategy for T_{fh} cells, $CXCR5^-$ T_{reg} cells, and T_{fr} cells in human blood.

Fig. S2. Variation of blood T_{fh} cells and total T_{reg} cells according to age.

Fig. S3. Sorting strategy for human blood naïve B cells, T_{fh} cells, $CXCR5^-$ T_{reg} cells, and T_{fr} cells.

Fig. S4. Representative plots of $CXCR5^-$ T_{reg} and T_{fr} cell suppression curves for coculture assay.

Fig. S5. Expression of CD45RO, CD45RA, and CD31 by human blood T_{fh} and T_{fr} cells.

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Science Immunology

Human blood T_{fr} cells are indicators of ongoing humoral activity not fully licensed with suppressive function

Valter R. Fonseca, Ana Agua-Doce, Ana Raquel Maceiras, Wim Pierson, Filipa Ribeiro, Vasco C. Romão, Ana Rita Pires, Susana Lopes da Silva, João Eurico Fonseca, Ana E. Sousa, Michelle A. Linterman and Luis Graca

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Suppressing Sjögren syndrome

T follicular regulatory (T_{fr}) cells regulate antibody production in the germinal center, yet individuals with the autoimmune disease Sjögren syndrome have increased numbers of circulating T_{fr} cells compared with healthy individuals. Fonseca *et al.* compared blood T_{fr} cells with tissue T_{fr} cells and found that blood T_{fr} cells were phenotypically distinct from their tissue counterparts. Moreover, blood T_{fr} cells did not preferentially suppress humoral responses and had a naïve-like phenotype. These cells were not thymically derived but were generated during germinal center responses, exiting the tissue to enter the blood. These data explain why increased number of blood T_{fr} cells does not correlate with increased suppression potential and suggest that, instead, increased numbers of blood T_{fr} cells indicate ongoing humoral activity.

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Science Immunology

Supplementary Materials for

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This PDF file includes:

Supplementary Materials

Patient	Age	Gender	pSS / sSS	ESSDAI	Treatment
SS1	71	F	Secondary (RA)	-	No
SS2	31	F	Secondary (SLE)	-	No
SS3	24	F	Primary	0	No
SS4	39	F	Primary	-	No
SS5	50	F	Primary	10	No
SS6	57	F	Primary	18	No
SS7	39	F	Secondary (SLE)	-	PDN + HCQ
SS8	27	F	Primary	16	No
SS9	20	F	Primary	0	No
SS10	66	F	Primary	0	No
SS11	57	F	Primary	0	No
SS12	62	F	Primary	0	HCQ
SS13	33	F	Primary	0	No
SS14	50	F	Primary	0	No
SS15	67	F	Primary	1	No
SS16	66	F	Secondary (PBC)	-	PDN + HCQ
SS17	58	F	Primary	11	PDN
SS18	17	F	Primary	3	No
SS19	49	F	Secondary (SLE)	7	PDN + HCQ
SS20	46	F	Primary	0	No
SS21	78	F	Primary	5	PDN + HCQ
SS22	40	M	Primary	2	No
SS23	79	F	Primary	6	No
SS24	69	F	Primary	11	PDN
SS25	55	F	Primary	-	PDN + HCQ

Table S1: Clinical characteristics of 25 patients with primary (pSS) and secondary (sSS) Sjögren syndrome. RA, rheumatoid arthritis. SLE, systemic lupus erythematosus. PBC, primary biliary cirrhosis. ESSDAI, EULAR Sjögren's syndrome disease activity index. PDN, prednisolone. HCQ, hydroxychloroquine.

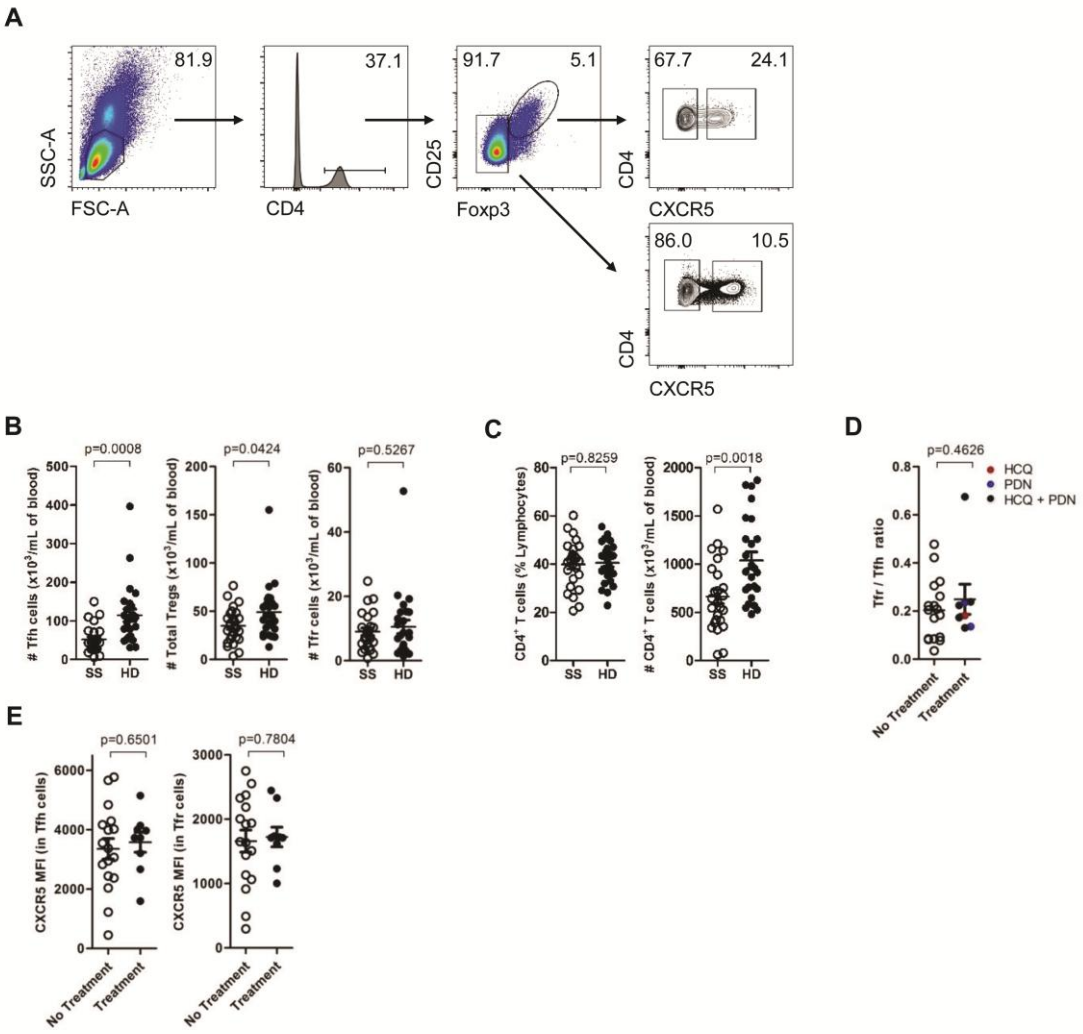

Figure S1: Gating strategy for T_{fh} cells, CXCR5 T_{reg} cells, and T_{fr} cells in human blood. **(A)** Gating strategy for identification and analysis of conventional T cells (Tconv), Tfh cells, $CXCR5^-$ Tregs and $CXCR5^+$ Tfr in human peiprheral blood. **(B)** Absolute numbers of Tfh cells, total Tregs and Tfr cells (cells/mL of blood) in peripheral blood of Sjögren's syndrome (SS) patients and healthy donors (HD) (n = 25, Student t-test). **(C)** Frequency and absolute numbers (cells/mL of blood) of $CD4^+$ T cells in peripheral blood of SS patients and HD (n = 25, Student t-test). **(D)** Blood Tfr/Tfh ratio in SS patients accordingly with treatment regimen (HCQ, hydroxychloroquine; PDN, prednisolone) (n = 25, Student t-test). **(E)** CXCR5 MFI in Tfh (left) and Tfr cells (right) in SS patients accordingly with treatment (n = 25, Student t-test). Error bars represent SEM.

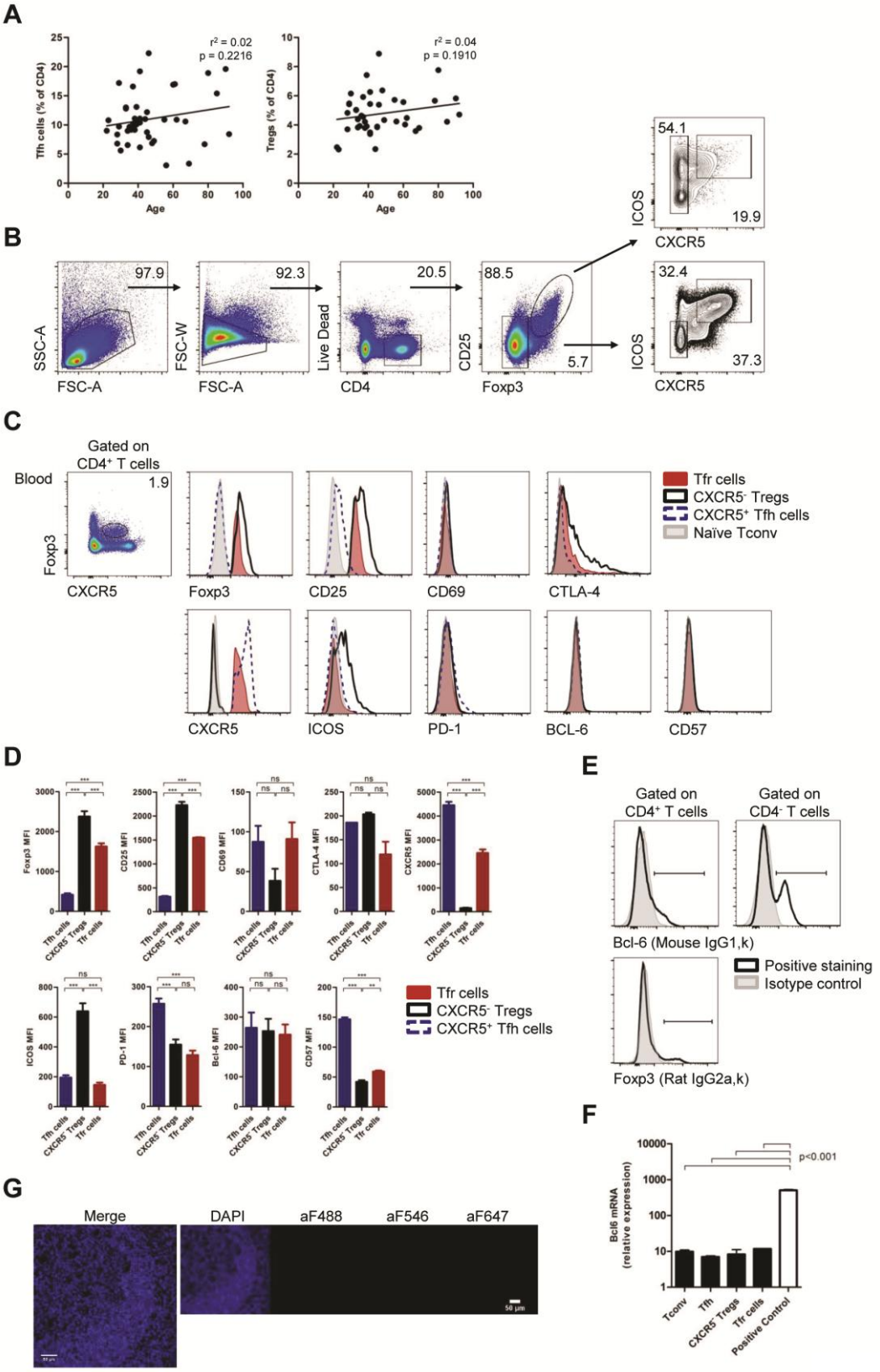

Figure S2: Variation of blood T_{fh} cells and total T_{reg} cells according to age.

(A) Variation of blood Tfh cells (left) and total Tregs (right) frequency

accordingly to age (age range: 22 – 92 years old) (n = 42, linear regression). **(B)** Gating strategy for identification and analysis of Tconv, Tfh cells, $CXCR5^-$ Tregs and $CXCR5^+$ Tregs in human tonsils. **(C)** Expression of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1 Bcl-6, and CD57 by Tfh cells (blue), $CXCR5^-$ Tregs (black) and $CXCR5^+$ Tfr cells (red) in adult blood. Naïve CD4+ T cells were used as control (gray).Representative plots from 42 healthy volunteers. **(D)** MFI of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1 Bcl-6, and CD57 by Tfh cells (blue), $CXCR5^-$ Tregs (black) and Tfr cells (red) in adult blood (**$p<0.01$, ***$p<0.001$, n = 6, one-way ANOVA with post-test Turkey's multiple comparison) **(E)** Isotype controls for intracellular Bcl-6 and Foxp3 staining by flow cytometry. **(F)** Relative expression of Bcl-6 by sorted Tconv, Tfh cells, CXCR5- Tregs and Tfr cells from blood, by real-time RT-PCR. Gene expression normalized to housekeeping genes (B2M, G6PD and ACTB) (n = 2, each with technical duplicates, Student t-test). $CD38^{hi}IgD^-$ germinal center B cells sorted from human tonsils were used as positive control. **(G)** Negative control for immunofluorescence microscopy, merge (left) and composite of the four immunofluorescence channels (right). After paraffin removal and antigen retrieval by heat sections of formalin-fixed paraffin-embedded human tonsil were stained with Alexa-Fluor 488 (anti-mouse), Alexa-Fluor 546 (anti-rabbit) and Alexa-Fluor (anti-Rat) secondary antibodies, without primary antibodies. DAPI was used as nuclei counterstaining. Unspecific binding of secondary antibodies and cross-reactivity between secondary antibodies were excluded. Error bars represent SEM. (ns = not significant).

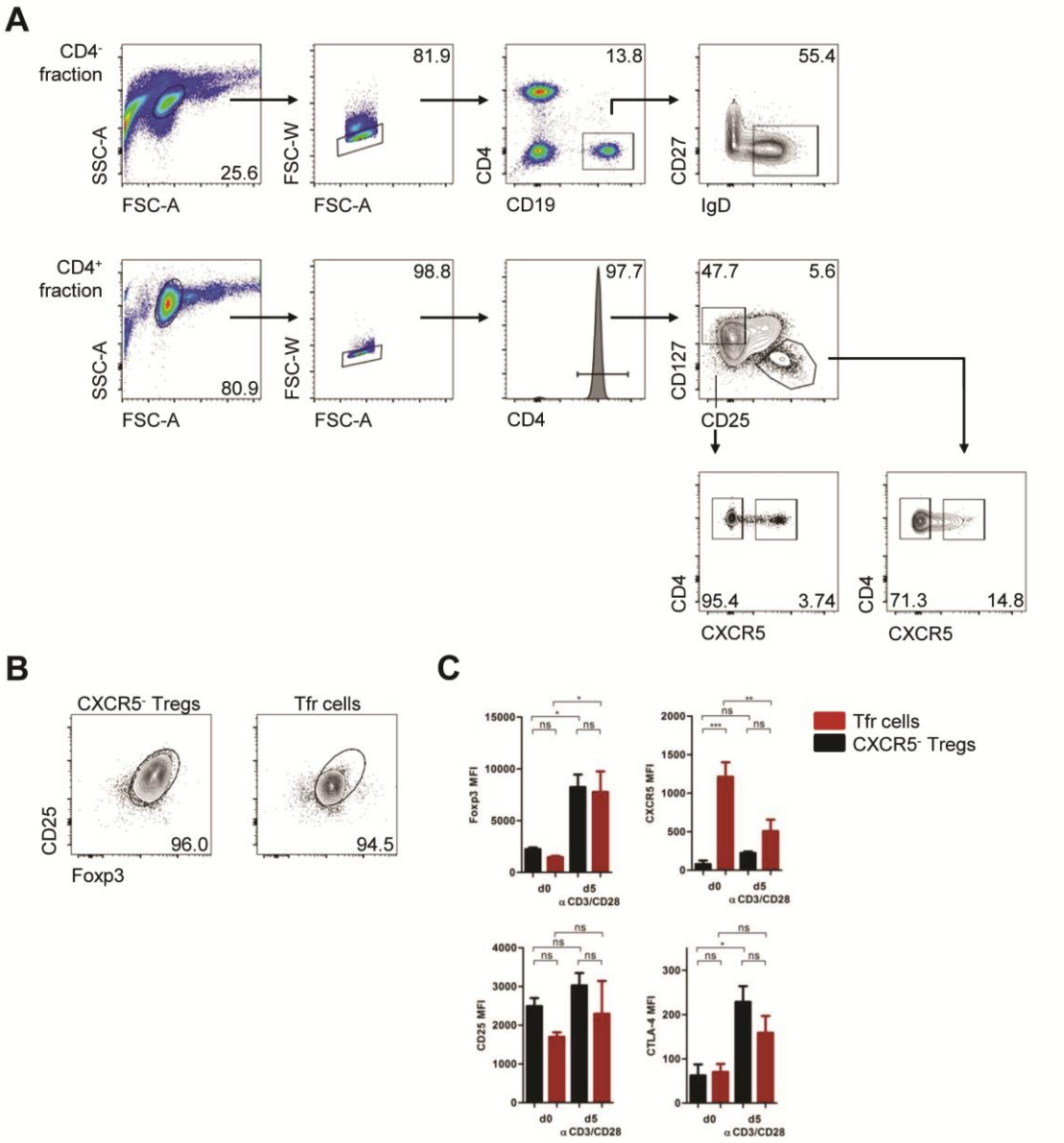

Figure S3: Sorting strategy for human blood naïve B cells, T_{fh} cells, CXCR5$^-$ T_{reg} cells, and T_{fr} cells. (A) Sorting strategy for FACS-sort of CXCR5$^+$CD25 CD127$^+$CD4$^+$ Tfh cell, CXCR5$^-$CD25$^-$CD127$^+$CD4$^+$ Tconv cell, CXCR5$^+$CD25$^+$CD127$^-$CD4$^+$ Tfr cells, CXCR5$^-$CD25$^+$CD127$^-$CD4$^+$ Tregs, and CD27$^-$IgD$^+$CD19$^+$ naïve B cell populations from peripheral blood (buffy-coats). **(B)** Purity of FACS-sorted CXCR5$^+$CD25$^+$CD127$^-$ CD4$^+$ Tfr cells, CXCR5$^-$CD25$^+$CD127$^-$CD4$^+$ Tregs. **(C)** MFI of Foxp3, CD25, CD69, CTLA-4, CXCR5, ICOS, PD-1 Bcl-6, and CD57 by CXCR5$^-$ Tregs (black) and Tfr cells (red) by sorted CXCR5$^-$ Tregs and Tfr cells at baseline (d0) and after 5 days of *in*

vitro culture under αCD3/αCD28 (1 μL/well) stimulation in adult blood (*p<0.05, **p<0.01, ***p<0.001, n = 3, one-way ANOVA with post-test Turkey's multiple comparison). Error bars represent SEM. (ns = not significant).

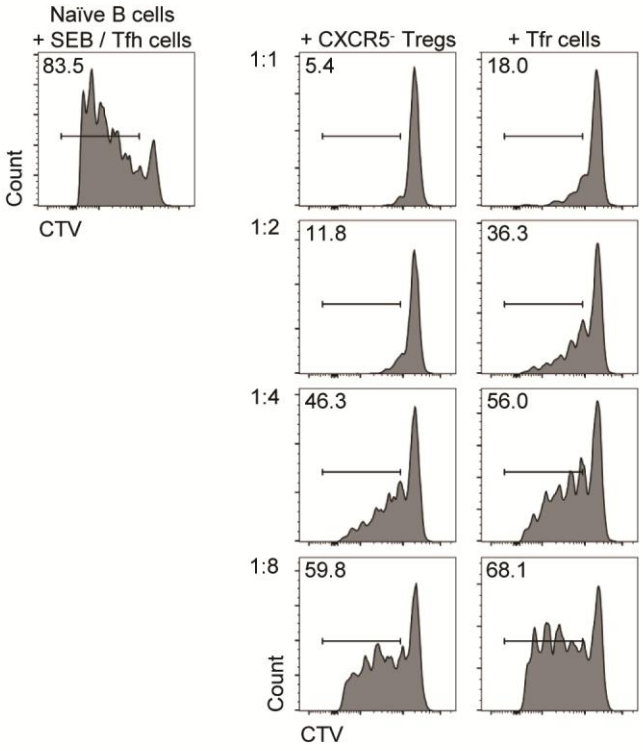

Figure S4: Representative plots of CXCR5^{-} T_{reg} and T_{fr} cell suppression curves for coculture assay. Proliferation of Tfh cells by CTV dilution without Tregs or in the presence of either CXCR5^{-} Tregs or Tfr cells (n = 1, each with technical triplicates), at 1:1, 1:2, 1:4 and 1:8 suppressor cell to responder cell ratios.

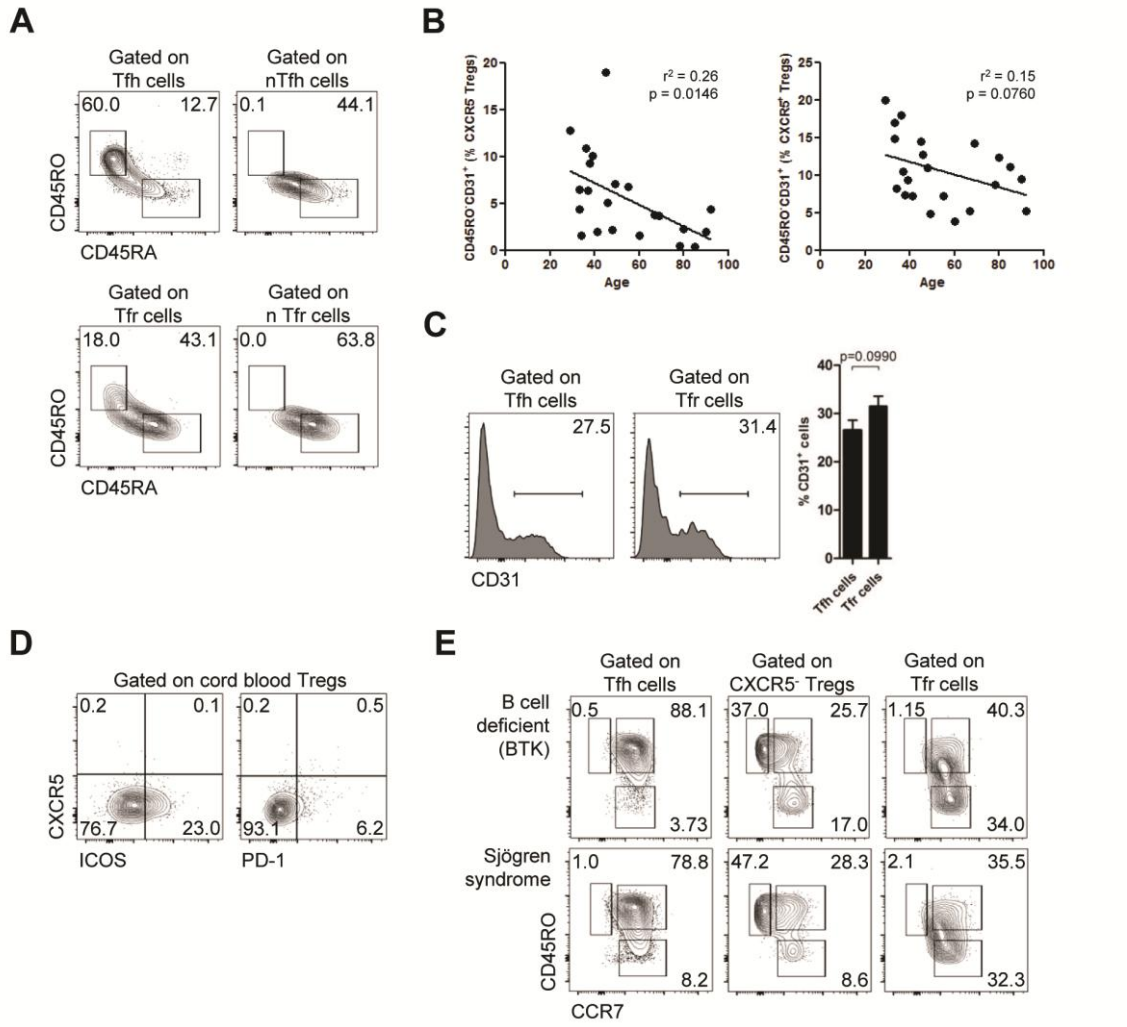

Figure S5: Expression of CD45RO, CD45RA, and CD31 by human blood T_{fh} and T_{fr} cells. (A) Expression of CD45RO and CD45RA in adult blood by total Tfh cells and Tfr cells (left) and by $CCR7^{+}CD45RO^{-}$ naïve Tfh cells (nTfh cells) and by $CCR7^{+}CD45RO^{-}$ naïve Tfr cells (nTfr cells) (right). Representative plots from 22 healthy donors. **(B)** Variation of $CD45RO^{-}CD31^{+}$ naïve Tfr cells and $CXCR5^{-}$ Tregs frequency in blood accordingly to age (n = 22, linear regression). **(C)** Expression of CD31 by Tfh and Tfr cells in adult blood of healthy donors. Representative plots (left) and pooled data (right) (n = 22, Student t-test). **(D)** Expression of CXCR5, ICOS and PD-1 by cord blood $Foxp3^{+}CD25^{+}$ Tregs. Representative plots from 3 healthy newborns. **(E)** $CD45RO^{+}CCR7^{-}$ effector-memory (EM), $CD45RO^{+}CCR7^{+}$ central-

memory (CM) and $CD45RO^{-}CCR7^{+}$ naïve subsets of Tfr cells and $CXCR5^{-}$ Tregs in peripheral blood of SS and BTK patients. Representative plots of 25 SS patients and 5 BTK patients. Error bars represent SEM.

Article type : Full Length

Blood T Follicular Regulatory Cells / T Follicular Helper Cells ratio Marks Ectopic Lymphoid Structure Formation and PD-1^{+}ICOS^{+} T Follicular Helper Cells Indicate Disease Activity in Primary Sjögren's Syndrome

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Running Head: Blood Tfr / Tfh ratio Marks ELS Formation and PD-1+ICOS+ Tfh cells Indicate Disease Activity in Sjögren's Syndrome

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ABSTRACT

Objectives: To investigate whether the balance of blood T follicular helper (Tfh) and Foxp3+ T follicular regulatory (Tfr) cells can inform on ectopic lymphoid neogenesis and disease activity in primary Sjögren's syndrome (SS).

Methods: We prospectively recruited 56 patients with clinical suspicion of SS, 16 of which subsequently fulfilled American European Consensus Group primary SS classification criteria and were compared with 16 patients with non-Sjögren sicca syndrome. Paired blood and minor salivary gland (MSG) biopsies were analysed to study Tfh subsets and Tfr cells in both compartments.

Results: Primary SS patients had normal Tfh cell counts in peripheral blood, however, blood activated PD-1^{+}ICOS^{+} Tfh cells were strongly associated with disease activity assessed by EULAR SS Disease Activity Index (r=0.85, p=0.0008). Conversely, blood Tfr/Tfh ratio indicated ectopic lymphoid structure formation in MSG, being strongly associated with B, CD4^{+} T and PD-1^{+}ICOS^{+} T cell infiltration in MSG, and especially increased in patients with focal sialadenitis (FSA). Further analysis showed that blood Tfr/Tfh ratio allowed discrimination between SS patients and healthy donors with excellent accuracy and was a strong predictor of SS diagnosis (OR=12.96, p=0.028) and

FSA presence (OR=10.0, p=0.022) in patients investigated for sicca symptoms, thus highlighting the potential clinical value of this marker.

Conclusions: Blood Tfr/Tfh ratio and PD-1^{+}ICOS^{+} Tfh cells constitute potential novel biomarkers for different features of primary SS. While blood Tfr/Tfh ratio is associated with ectopic lymphoid neogenesis, activated Tfh cells indicate disease activity.

Keywords

Sjögren's syndrome; Ectopic lymphoid structures; T follicular helper cells; T follicular regulatory cells.

INTRODUCTION

Sjögren's syndrome (SS) is characterized by lymphocytic infiltration of salivary and lachrymal glands, leading to xerostomia and keratoconjunctivitis sicca (1–3). However, like in other autoimmune diseases, disease heterogeneity remains a hurdle challenge to the development of better therapeutic approaches. Disease manifestations are mediated by complex mechanisms involving innate type I IFN signature and autoantibodies produced during dysregulated adaptive immune responses (2). Ectopic lymphoid structures (ELS) (4–6) found in salivary and lachrymal glands are a major site of autoantibody production (2,7,8). These B-T lymphocyte aggregates define the so-called focal sialadenitis (FSA), the histological hallmark of SS, and have prognostic significance (9,10). Nevertheless, many patients fulfil classification criteria for SS with almost normal salivary gland histology, consistently with a highly heterogeneous disease. Whether clinical heterogeneity could be explained by different

immunopathogenic mechanisms is unknown. However, it is reasonable to hypothesize that patients with FSA, especially those with ectopic germinal centre (GC) formation, constitute a subgroup of patients more suitable for therapies targeting B-T interactions.

Dysregulated GC reactions in secondary and tertiary lymphoid organs underlie the generation of self-reactive autoantibodies and many aspects of autoimmune diseases (4,5). GC reactions are orchestrated mainly by T follicular helper (Tfh) and T follicular regulatory (Tfr) cells. Tfh cells provide cognate help to B cells, thus promoting their clonal selection and affinity maturation (11). Conversely, Tfr cells regulate and limit the GC reaction assuring antigen-specific antibodies are produced (12–16). While the precise mechanisms of Tfr cell functions are not fully understood, unbalanced Tfh and Tfr cell responses may prompt antibody-mediated autoimmune diseases.

We have previously described an increased Tfr/Tfh ratio in peripheral blood of SS patients (17). Furthermore, we demonstrated that blood Tfr cells are immature and not yet fully specialized in suppressing humoral responses. However, the relationship between blood and tissue Tfr cells remains elusive in patients with immune-mediated diseases. Herein we investigated the potential role of blood Tfr and Tfh cells as biomarkers of ectopic lymphoid activity in the target organ of SS.

METHODS

Human samples

We recruited patients referred to the Rheumatology and Metabolic Bone Diseases Department of Hospital de Santa Maria, for minor salivary gland (MSG) biopsy due to clinical suspicion of SS. Fresh blood samples were collected on the day of MSG biopsy. MSG tissue was divided for routine diagnostic purposes and for flow cytometry and microscopy. We excluded patients treated with biologic drugs, disease modifying anti-rheumatic drugs (DMARDs) other than hydroxychloroquine or more that 7.5 mg per day of prednisolone equivalent, diagnosed with an infectious disease or who had received any vaccine in the previous month, and those with exclusion criteria for SS (**Fig. S1**) (18). Patients were classified as having primary SS if they fulfilled American European Consensus Group (AECG) 2002 classification criteria (n=16) (18), whereas those with sicca symptoms and no evidence of an inflammatory rheumatic disease were classified as non-Sjögren sicca syndrome and used as appropriate controls (n=16) (**Fig. S1, Table 1, S1**). Patients with secondary SS (n=6) or with clinical diagnosis of primary SS but not fulfilling AECG criteria (n=5) were excluded from analysis to standardize populations. Disease activity was evaluated by the European League Rheumatism SS disease activity score (ESSDAI) (19). Routine serum autoantibodies, c-reactive protein (mg/dL), erythrocyte sedimentation rate (mm) and serum electrophoresis gamma-fraction (g/dL) levels closest to blood collection were used. Fresh blood was collected from age and gender-matched healthy controls. Tonsils were obtained from children submitted to tonsillectomy due to tonsil hypertrophy (n=6). Children with any clinical condition, under any drug treatment, or with chronic tonsillitis were excluded. Blood samples were collected in EDTA coated tubes.

This study was approved by the Lisbon Academic Medical Center Ethics Committee (reference number 505/14). Informed consent was obtained from all study participants.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-gradient medium (Histopaque-1077, Sigma-Aldrich) using SepMate tubes (StemCell Technologies). Lymphocytes from tonsils were also isolated by Ficoll-gradient medium after mechanical disruption. To study human MSG biopsies, we first optimized the experimental protocol to ensure that enzymatic digestion of tissue had negligible impact on cellular markers (**Fig. S3A**). Then, we used the optimized protocol to obtain a cell suspension from MSG. Briefly, MSG fragments were incubated at 37ºC with Liberase TM 0.1 mg/mL and DNAse I 0.1 mg/mL in RPMI medium followed by mechanical digestion to obtain a cell suspension. To normalize cell counts, MSG were weighed before digestion (**Fig. S3B**). Antibodies used for flow cytometry are described in **Table S2**. For Bcl-6, and Foxp3 intracellular staining, Foxp3 Fix/Perm Kit (eBioscience) was used accordingly to manufacturer instructions. For cell viability staining, Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used. Flow cytometry analysis was performed in a LSR Fortessa (BD Biosciences) and further analyzed with FlowJo v10 software (TreeStar).

Immunohistochemistry microscopy

MSG were fixed in 10% buffered formaldehyde, embedded in paraffin wax and sectioned into 3µm sequential sections. Sections were stained after deparaffinisation, pretreatement with Ultra CC1 (Ventana Medical Systems, USA), antigen retrieval and endogenous peroxidase blocking using Benchmark machine. Sections were

immunohistochemically stained with anti-human CD20 (L26, DAKO), anti-human CD21 (EP3093, Roche), anti-human Bcl-6 (LN22, Leica), anti-human CXCR5-Alexa-Fluor 488 (J252D4, BioLegend), and anti-human Foxp3 (236A/E7, eBiosciences). The sections were then treated with peroxidase-labelled secondary antibody and visualized with the chromogen DAB solution and/or universal AP Red. Hematoxylin was used as counterstaining. Images were acquired with NanoZoomer-SQ (Hamamatsu, Japan) using a 20x objective with a numerical aperture of 0.75. Images were further analyzed using NDP.view software. Number of CXCR5 and Foxp3 double positive cells were counted manually. Routine hematoxylin and eosin staining was used to define histological diagnosis in MSG: Normal, section with no pathological findings; LI, sections with unspecific lymphocytic infiltration; and FSA, sections with ≥1 dense aggregate of ≥50 lymphocytes per $4mm^2$ of tissue located in perivascular or periductal locations (**Fig. S3G**) (20).

Statistical analysis

Unpaired Student T-test, one-way ANOVA with post-test Turkey's multiple comparison, and two-way ANOVA with post-test Bonferroni's multiple comparison were used as described. Normality of data was assessed by Shapiro-Wilk test. Variance across groups was assessed with Levene's and Brown–Forsythe's tests and, where different, Welch correction of T-test or Mann Whitney U test were preferred. Pearson correlation and linear regression were also conducted for some data. For categorical variables Fisher's exact test was used. Logistic regression and receiver operating characteristic (ROC) analysis were conducted to study the association of Tfr/Tfh ratio with SS and FSA and the cut-off with the best discriminative value was determined.

Results are presented as mean ± SD. P values of less than 0.05 were considered statistically significant. GraphPad Prism v5 and Stata v.12.1 were used for statistical and graphical analysis. Correlation heatmaps were computed in R studio v3.1.2.

RESULTS

Blood activated PD-1$^+$ICOS$^+$ Tfh cells correlate with SS disease activity

We previously reported that SS patients had normal CXCR5$^+$CD45RO$^+$ Tfh cells, increased CXCR5$^+$Foxp3$^+$ Tfr cells and increased Tfr/Tfh ratio in peripheral blood (17). To refine our analysis, we now studied only patients with primary SS, and we further characterized blood Tfh cell subsets in those patients. Consistently, primary SS patients showed an increased frequency of blood Tfr cells and an increased blood Tfr/Tfh ratio compared to healthy donors (**Fig. 1A, B**). Interestingly, while primary SS patients had decreased absolute number of all CD4$^+$ T cell subsets, in line with their CD4$^+$ T cell lymphopenic state, the absolute number of Tfr cells were not different when compared to healthy donors (**Fig. S2A-D**), suggesting that the Tfr cell subset is indeed over-represented in these patients. We did not find a significant increase of activated PD-1$^+$ICOS$^+$ Tfh cells or PD-1$^+$CXCR3$^-$ GC-like Tfh cells in SS patients (**Fig. 1C, D**) (21–25). Contrary to previous reports (26), SS patients had a normal distribution of Tfh1, Tfh2 and Tfh17-like cells (**Fig. 1E, S2E**). To investigate whether follicular T cell subsets correlated with autoantibodies, inflammatory markers and disease activity we conducted correlation heatmaps. We found that blood Tfr/Tfh ratio and PD-1$^+$ICOS$^+$ Tfh cells significantly correlated with the presence of anti-SSA/Ro60 (r=0.580, p=0.0298) and anti-SSA/Ro52 (r=0.558, p=0.047), respectively (**Fig. 1F, G**). While most correlations were barely significant, we found a very strong association between

frequency of activated PD-1$^+$ICOS$^+$ Tfh cells and disease activity (ESSDAI, r=0.855, p=0.0008) (**Fig. 1F, G**). Interestingly, blood Tfr/Tfh ratio and PD-1$^+$ICOS$^+$ Tfh cells consistently correlated in opposite directions with the variables analysed, albeit not always reaching statistical significance (**Fig. 1F**).

Blood Tfr/Tfh ratio identifies pathological lymphocytic infiltration in SS target organ

As the Tfr/Tfh ratio may predict the outcome of GC reactions (12,27), we hypothesized that in patients with autoimmune diseases with abnormal GC reactions leading to autoantibody production may have an altered Tfr/Tfh ratio. As GC reactions take place in lymphoid tissues and ectopic (or tertiary) lymphoid structures, we studied MSG tissue biopsies from patients with primary SS and controls with non-Sjögren sicca syndrome (**Fig. S1 and Table S1**).

Sjögren's syndrome patients had an increased frequency and absolute number (per mg of tissue) of non-epithelial CD45$^+$ cells (**Fig. 2A**) and CD19$^+$ B cells (**Fig. 2B**). Loss of CXCR5 receptor following enzymatic digestion due to a tissue-intrinsic factor excluded direct analysis of tissue Tfh and Tfr cells (**Fig. S3A, C, D**). Nevertheless, as around 90% of PD-1$^+$ICOS$^+$ T cells in lymphoid tissues were CXCR5$^+$ Tfh cells, we assessed salivary gland infiltration by PD-1$^+$ICOS$^+$ T cells (**Fig. 2C, S3E**). We found a striking increased frequency and absolute number of infiltrating PD-1$^+$ICOS$^+$ T cells in SS patients (**Fig. 2C**). Although, it is likely that this cell population comprise tissue Tfh cells, we cannot exclude the existence of CXCR5$^-$PD-1$^+$ICOS$^+$ T cells, described as a specific type of helper T cells within ELS of rheumatoid arthritis patients (28). In both cases, our findings suggest that MSG tissue from primary SS patients host active T-

dependent humoral immune responses. To validate our results, we compared MSG analysis by flow cytometry with routine histological diagnosis of the same biopsies (**Fig. S3F**). Infiltration by $CD19^+$ B cells was found in all SS patients irrespectively of salivary gland morphology. Yet, only patients with ELS/FSA had a significantly higher infiltration by B cells as compared to MSG of patients with non-Sjögren sicca syndrome (**Fig. S3G**).

When we compared blood Tfh and Tfr populations with lymphocytic MSG infiltration of patients with SS, we found a strong correlation between blood Tfr/Tfh ratio and the presence of ELS in exocrine glands. Indeed blood Tfr/Tfh ratio was positively correlated with tissue infiltration by $CD4^+$ T cells (r=0.8475, p=0.0039), PD-$1^+$$ICOS^+$ T cells (r=0.8400, p=0.0180), and $CD19^+$ B cells (r=0.6748, p=0.0462) (**Fig. 2D, E**). Moreover, blood Tfr cells were also positively correlated with tissue $CD19^+$ B cells (r=0.6690, p=0.0488) and a positive trend was seen with other local populations (**Fig 2D**). Interestingly, despite not significantly, blood Tfh cells and Tfh cell subsets tended to negatively correlate with tissue infiltration by all inflammatory cells analysed, showing an opposite trend to blood Tfr cells and Tfr/Tfh ratio (**Fig 2D**). Taken together, these data suggest that blood Tfr cells and Tfr/Tfh ratio indicate pathological lymphocytic infiltration in the target organ of SS. On the other hand, blood activated PD-$1^+$$ICOS^+$ Tfh cells correlated with disease severity assessed by ESSDAI (**Fig. 1F**), but nor with the presence of FSA (**Fig.2D**). Indeed, the presence of FSA did not correlate with ESSDAI (**Fig. S4A**), as previously shown (29,30).

To test whether increased blood Tfr cells and Tfr/Tfh ratio observed in patients is due to selective exclusion of Tfr cells from ELS we directly access $CXCR5^+$$Foxp3^+$ Tfr cells infiltration in MSG biopsies by microscopy. MSG biopsies from patients with FSA were further studied to identify ELS based on the presence of organized $CD20^+$ B cell

aggregates and infiltration by $CD21^+$ follicular dendritic cells (**Fig. S4B, C**) (31). As, only one patient had FSA without infiltration by $CD21^+$ follicular dendritic cells we assumed that FSA was a good indicator for ELS in our cohort (Fig. S4B). Additionally, two patients with FSA were also found to have Bcl-6^+ ectopic GC formation (**Fig. S4B, C**). We found a substantial number of $CXCR5^+Foxp3^+$ Tfr cells within ELS in MSG of SS patients following a bimodal distribution, with a group of patients having a tissue Tfr density 10-fold greater that the other group (**Fig. 2F**). We therefore assessed whether the tissue density of Tfr cells correlated with disease-related parameters (Table S3). We found a strong correlation of tissue Tfr cells with $ICOS^+PD\text{-}1^+$ T cell infiltration (**Fig. 2G**). Overall our data show Tfr cells are not excluded from ELS in inflamed SS exocrine glands.

Blood Tfr/Tfh ratio as a marker of SS and focal sialadenitis

The presence of focal sialadenitis (FSA) on MSG biopsy is a major diagnostic and prognostic marker of primary SS (9,20,32), and one of the diagnostic criteria established by American European Consensus Group in 2002 (Vitali C, et al. Ann Rheum Dis 2002). As blood Tfr/Tfh ratio was increased in SS patients compared to healthy donors and associated with MSG lymphocytic infiltration we wondered whether this ratio could aid in the identification of patients with SS and ectopic lymphoid activity (typical FSA).

The blood Tfr/Tfh ratio was significantly higher in SS patients compared to healthy donors and patients with non-Sjögren sicca syndrome (**Fig. 3A**). This ratio was a significant predictor of SS when compared to healthy donors, with an increase of 4.97 in the odds of having SS for every decimal increase in the Tfr/Tfh ratio (AUC=0.82, p=0.008) (**Fig. 3B, C**). Through ROC analysis, we found that a cut-off of ≥0.278

correctly classified 87.1% of patients with 100% specificity and 73.3% sensitivity. Compared to non-Sjögren sicca syndrome, Tfr/Tfh ratio tended to associate with SS diagnosis (OR=1.93, p=0.099, AUC=0.716), with the best cut-off to diagnose SS being a blood Tfr/Tfh ratio ≥0.3009 **(Fig. 3B, C**). Thus, patients with sicca symptoms and a Tfr/Tfh ratio ≥0.3009 had 13 times the odds of having SS, with very high specificity (90.9%) but moderate sensitivity (56.3%) (**Fig. 3C**).

Importantly, blood Tfr/Tfh ratio was significantly increased in patients with FSA, compared to those with normal histology or mild unspecific inflammation, irrespective of diagnosis (**Fig. 3D**). Moreover, it significantly predicted the result of MSG biopsy (FSA vs. no-FSA), with an increase in the odds of having FSA of 2.03 per each decimal increase (AUC 0.793, p=0.047),(**Fig. 3C, E**). Values of blood Tfr/Tfh ratio ≥0.302 were significantly associated with FSA (OR=10, p=0.022, AUC=0.757), with good sensitivity (71.4%) and very good specificity (80%) **(Fig. 3C**).

DISCUSSION

Our results show that blood activated Tfh cells and Tfr/Tfh ratio are associated, respectively, with disease activity and ectopic lymphoid structures in MSG, respectively, in primary SS. Indeed, there is a striking association between high Tfr/Tfh ratio and ectopic lymphoid activity in salivary glands, the target organ of this disease. In addition, circulating PD-$1^{+}$$ICOS^{+}$ Tfh cells seem to provide a different information, indicating disease activity and not ELS formation.

The relationship between circulating PD-$1^{+}$$ICOS^{+}$ Tfh cells with ESSDAI was an exciting finding. While, many studies have established that circulating activated Tfh cells are correlated with disease severity in autoimmunity (33–35), so far circulating Tfh

cells were not correlated with ESSDAI. Notably, other Tfh subsets, namely Tfh17-like cells identified in other immune-mediated diseases (35), were not altered in our cohort of primary SS patients., These results suggest that $PD1^+ICOS^+$ Tfh cells may be directly involved in the pathogenesis of SS, particularly in those patients with greater inflammatory activity and high ESSDAI scores. A recent study showed that ESSDAI is an independent predictor of lymphoma development in primary SS (36) thus raising the possibility that increased circulating $PD1^+ICOS^+$ Tfh cells are somehow related to the same mechanism that will culminate in hematologic malignancy.

The predictive value of Tfr cells, and especially the Tfr/Tfh ratio, regarding the diagnosis of primary SS and pathological lymphocytic infiltration in salivary glands may appear counterintuitive, given the regulatory role attributed to Tfr cells (12,13). However, we have recently shown that human blood Tfr cells remain immature and are generated prior to T-B interactions required for acquisition of follicle access and full regulatory function (17). The increased proportion of Tfr cells in peripheral blood of SS patients did not seem to be due to anatomic exclusion of Tfr cells from inflamed exocrine glands, as substantial numbers of Tfr cells were found within ELS containing B cells in the majority of patients. It is generally assumed that ELS are populated by lymphocytes formed outside these structures, in secondary lymphoid organs (4,6). Thereby, it is likely that Tfr cells result from enhanced differentiation in secondary lymphoid organs due to ongoing humoral responses. Our data suggest that accumulation of Tfr cells are not sufficient to restore tolerance in exocrine glands (where the first step of SS pathogenesis occur), although we did not directly address the presence of Tfr cells in ELS with and without GCs (3). A recent report claimed salivary gland epithelial cells induce Tfh cell differentiation from naïve $CD4^+$ T cells (37). While, it is not known

whether Tfr cell differentiation can also occur *in situ,* this could possibility provide an alternative explanation for the increased proportion of blood Tfr cells in SS.

The blood Tfr/Tfh ratio does seem to be a specific marker of SS and of ELS formation within salivary glands. Indeed we found that Tfr/Tfh ratio could discriminate between SS patients and healthy donors with excellent accuracy, with a specific cut-off above which the diagnosis of SS was almost certain. Even more importantly, this ratio was also helpful in predicting a diagnosis of primary SS and the presence of FSA in MSG biopsy in a group of patients investigated for sicca symptoms, thus highlighting the potential clinical value of this marker. The proposed cut-offs of blood Tfr/Tfh ratio above/equal 0.3009 and 0.302 were strong predictors of SS and FSA, respectively, and constitute promising tools for SS clinical evaluation. It should be noted that the specificity and sensitivity of Tfr/Tfh ratio to predict FSA and SS was remarkably high, in particular given the small number of patients studied. We anticipate that greater patient numbers may reinforce the significance of our findings, potentially establishing the usefulness of Tfr and Tfh quantification for primary SS patient stratification. This is particularly important both for diagnostic and therapeutic purposes as there are major unmet needs in these fields in primary SS (38). The potential to confirm diagnosis in patients who do not fulfil classification criteria, or to identify clusters of patients more prone to respond to targeted therapies are some of the applications that such a biomarker could have.

In this report, we decided to study fresh blood of patients at the time of diagnosis who were not under immunosuppressant drugs, in order to overcome potential drug effects on Tfh and Tfr cell subsets. Consequently, the studied cohort of patients was small, but uniform and consistent. Other limitations of our study included the fact that paired samples for every parameter were in some cases not available and the technical issue of

CXCR5 staining in salivary gland cell suspension, which precluded a direct assessment of tissue Tfr cells at a phenotypical and functional level. It also remains to be shown whether alterations of Tfr and Tfh subsets that we describe here are specific for SS or can be generalized to other immune-mediated inflammatory diseases, in particular the ones with ELS and autoantibody-mediated pathology.

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Figure Legends

Figure 1: Blood activated PD-1$^+$ICOS$^+$ Tfh cells indicate disease activity in primary Sjögren's syndrome. (A) Frequency of CXCR5$^+$CD45RO$^+$CD25$^-$Foxp3$^-$CD4$^+$ Tfh, CD25$^+$Foxp3$^+$CD4$^+$ Treg, and CXCR5$^+$CD25$^+$Foxp3$^+$CD4$^+$ Tfr cells in peripheral blood of primary Sjögren's syndrome (SS, n=16) and healthy donors (HD, n=16). Unpaired Student T-test with Welch's correction for variance. Representative plots (left) and pooled data (right). **(B)** Blood Tfr/Tfh ratio in SS (n=16) and HD (n =16). Unpaired Student T-test with Welch's correction for variance. **(C)** Frequency of PD-1$^+$ICOS$^+$ Tfh cells in peripheral blood of SS (n=14) and HD (n=13). Unpaired Student T-test with Welch's correction for variance. Representative plots (left) and pooled data (right). **(D)** Frequency of PD-1$^+$CXCR3$^-$ Tfh cells in peripheral blood of SS (n=14?) and

HD (n=13). Unpaired Student T-test. Representative plots (left) and pooled data (right). **(E)** Distribution of $CCR6^+CXCR3^-$ Tfh17-like cells, $CCR6^-CXCR3^+$ Tfh1-like cells, and $CCR6^-CXCR3^-$ Tfh2-like cells in peripheral blood of SS (n=16) and HD (n=16). Unpaired Student T-test . **(F)** Heatmap representation of correlation between peripheral blood Tfh cells, PD-1^+ICOS^+ Tfh cells, PD-1^+CXCR3^- Tfh cells, Tfr cells, $CXCR5^-$ Treg/Tfh ratio, and Tfr/Tfh ratio and serum autoantibodies titers (antinuclear antibodies (ANA), anti-SSA/Ro52, anti-SSA/Ro60, anti-SSB), rheumatoid factor (RF, IU/mL), serum electrophoresis gamma-fraction (γ-fraction, g/dL), C-reactive protein (CRP, mg/dL), erythrocyte sedimentation rate (ESR, mm/1^{st} hour) and disease activity measured by European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI), in SS patients (n = 16, Pearson coefficient, r). **(G)** Correlation between peripheral blood Tfr/Tfh ratio and serum anti-SSA/Ro60 titers (left); correlation between blood PD-1^+ICOS^+ Tfh cells and serum anti-SSA/Ro52 (centre) and ESSDAI (right) (n = 16, linear regression with interpolated 95% confidence interval curves). Bars on scatterplots represent SEM. On linear regression plots, dashed line represent interpolated 95% confidence interval.

Figure 2: Blood Tfr/Tfh ratio identifies pathological lymphocytic infiltration in Sjögren's syndrome target organ. (A) Frequency and absolute numbers of $CD45^+$ hematopoietic cells in minor salivary gland biopsies (MSG) of primary Sjögren's syndrome patients (SS, n=14) and non-Sjögren sicca syndrome (non-SSS) patients (n=6). Representative plots (left) and pooled data (right). Unpaired Student T-test. **(B)** Frequency and absolute numbers of $CD4^+$ T cells and $CD19^+$ B cells in MSG biopsies of SS (n=14) and non-SSS (n=6). Representative plots (left) and pooled data (right). Unpaired Student T-test with Welch's correction for variance. **(C)** Frequency and

absolute numbers of PD-1$^+$ICOS$^+$ T cells in MSG biopsies of SS (n=10) and non-SSS (n=6). Representative plots (left) and pooled data (right). Unpaired Student T-test with Welch's correction for variance. **(D)** Heatmap representation of correlation between peripheral blood Tfh cells, PD-1$^+$ICOS$^+$ Tfh cells, PD-1$^+$CXCR3$^-$ Tfh cells, Tfr cells, CXCR5$^-$ Treg/Tfh ratio, and Tfr/Tfh ratio (rows) and salivary gland infiltration by CD45$^+$ hematopoietic cells, CD4$^+$ T cells, ICOS$^+$PD-1$^+$CD4$^+$ T cells and CD19$^+$ B cells (columns), in primary SS patients (n=14, Pearson coefficient, r). **(E)** Correlation between peripheral blood Tfr/Tfh ratio with salivary gland infiltration by CD45$^+$ hematopoietic cells, CD4$^+$ T cells, ICOS$^+$PD-1$^+$CD4$^+$ T cells and CD19$^+$ B cells (n=14, linear regression). **(F)** Identification of CXCR5$^+$Foxp3$^+$ Tfr cells (arrows on extreme right image) within FSA containing CD20$^+$ B cells in MSG biopsies of primary SS patients by immunohistochemistry. Extreme left image shows FSA containing B cells (CD20 in brown) (x100, digital zoom). The three images on right show double immunohistochemistry for CXCR5 (purple/red) and Foxp3 (brown). The squares in each image are amplified successively on the right (x100, x200, x400, digital zoom). Representative sections (left) and pooled data (right) (n=16). Bars on scatterplots represent SEM. On linear regression plots, dashed line represent interpolated 95% confidence interval.

Figure 3: Blood Tfr/Tfh ratio as a marker of primary Sjögren's syndrome and focal sialadenitis. (A) Blood Tfr/Tfh ratio in Sjögren's syndrome (SS, n=16) and non-Sjögren sicca syndrome (non-SSS, n=11) patients and healthy donors (HD, n =16). Unpaired Student T-test with Welch's correction for variance was used except when variance was not significantly different between groups (non-SSS vs. HD comparison). One-way ANOVA comparison across groups represented on top. **(B)** Receiver

operating characteristic (ROC) curve for prediction of SS diagnosis (vs. HD and vs. non-SSS) based on Tfr/Tfh ratio. AUC, area under the curve. **(C)** Odds-ratio (OR), p-value and AUC of logistic regression models predicting SS diagnosis (vs. HD and vs. non-SSS) and focal sialadenitis (FSA, vs. normal histology or unspecific lymphocytic infiltration, LI) based on Tfr/Tfh ratio as a continuous variable or as specific cut-offs. Tfr/Tfh ratio transformed by 1 decimal place ($*10^1$) for better interpretation of the OR. Percentages of sensitivity, specificity and correct classification of patients based on given cut-offs. **(D)** Blood Tfr/Tfh ratio of patients with sicca symptoms undergoing minor salivary gland biopsy, with focal sialadenitis (FSA, n=7) and normal histology or unspecific lymphocytic infiltration (LI, n=20). Mann-Whitney U test used due to skewed distribution of values and non-different variance according to Brown–Forsythe's test. **(E)** Receiver operating characteristic (ROC) curve for prediction of FSA diagnosis (vs. normal/LI) based on Tfr/Tfh ratio. AUC, area under the curve.

Tables

Table 1: Summary of demographic characteristics of Sjögren's syndrome patients, non-Sjögren sicca syndrome patients, and healthy donors.

	Primary SS n = 16	**Non-SSS** n = 16	**Healthy donors** n = 16	**p-value**
Age, y	52.3 ± 17.7	47.3 ± 14.9	48.3 ± 19.7	ns
Gender (f / m), n	15 / 1	15 / 1	11 / 5	ns
Disease duration, y	4.8 ± 6.5	-	-	N/A
ESSDAI	3.8 ± 5.0	-	-	N/A
Extraglandular involvement, n (%)	12 (75)	-	-	N/A
Prednisolone (≤5mg/d), n (%)	5 (31)	0	-	0.0434
Hydroxychloroquine, %	3 (19)	0	-	ns
ANA positive, n (%)	15 (94)	2 (13)	-	<0.0001
Anti-SSA positive, n (%)	15 (94)	0		<0.0001
Anti-SSA/Ro52 titer, UQ	7824 ± 15430	-	-	N/A
Anti-SSA/Ro60 titer, UQ	8728 ± 6079	-	-	N/A
Anti-SSB positive, n (%)	10 (63)	0		<0.0001
Anti-SSB titer, UQ	1206 ± 4236	-	-	N/A
RF positive, n (%)	8 (50)	1 (6)		0.0024
RF titer, UI/mL	45.7 ± 26.1	18.7 ± 0.0	-	N/A
CRP, mg/dL	0.53 ± 0.58	0.52 ± 0.78	-	ns
ESR, mm	43.7 ± 25.6	22.1 ± 16.3	-	0.010
γ-fraction, g/dL	1.76 ± 0.58	1.07 ± 0.31	-	0.002
MSG histology, n (%)				
Focal sialadenitis	7 (44)	0	-	0.0068
Non-specific lymphocytic infiltrate	6 (38)	5 (31)	-	ns
Normal	3 (19)	11 (69)	-	0.0113

Supplementary Information

Table S1: Demographic and clinical characteristics of primary Sjögren's syndrome (SS) and non-Sjögren sicca syndrome (non-SSS) patients.

Table S2: Antibodies used for flow cytometry.

Table S3: Correlation between absolute number of Tfr cells per mm^2 of minor salivary gland tissue section (Fig. 2F) wit clinical and laboratory parameters, and minor salivary gland infiltration by $CD45^+$, $CD4^+$, $CD19^+$ and PD-1^+ICOS^+ lymphocytes. (n=16, Pearson coefficient, r).

Figure S1: Flowchart of patient recruitment and selection. DMARDs, disease modifying anti-rheumatic drugs; TNF, tumor necrosis factor; AECG, American-European Consensus Group criteria for Sjögren's syndrome.

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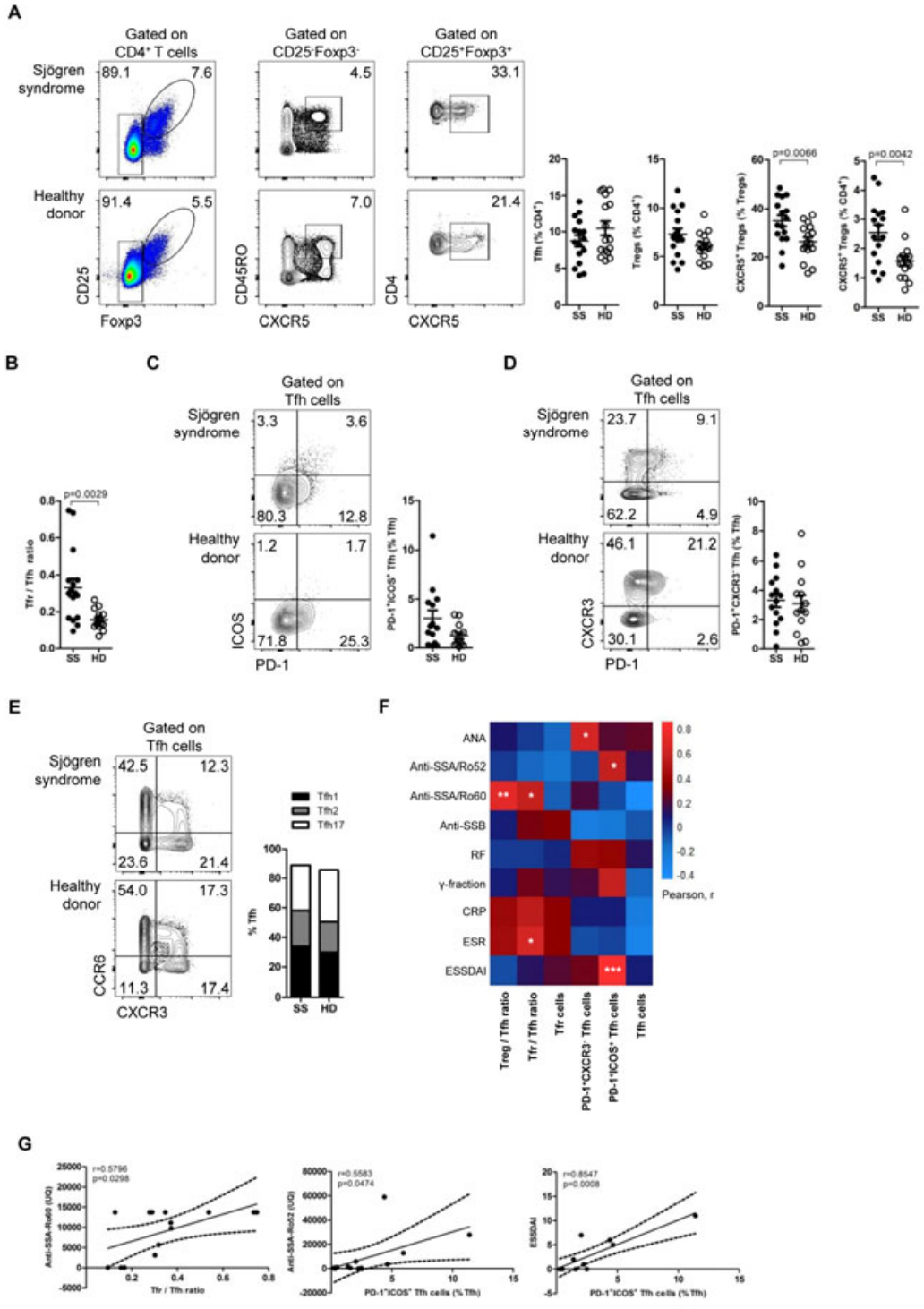

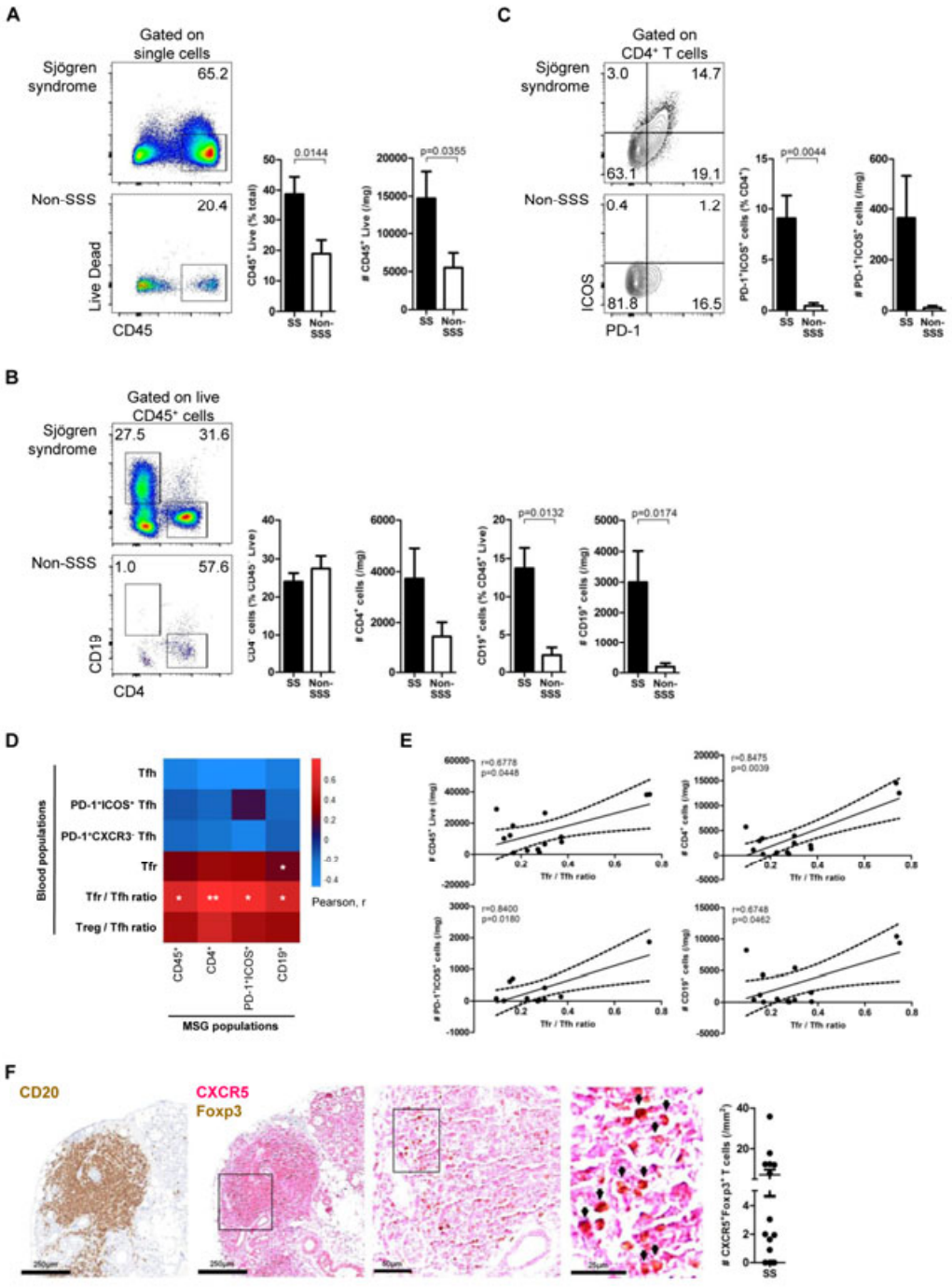

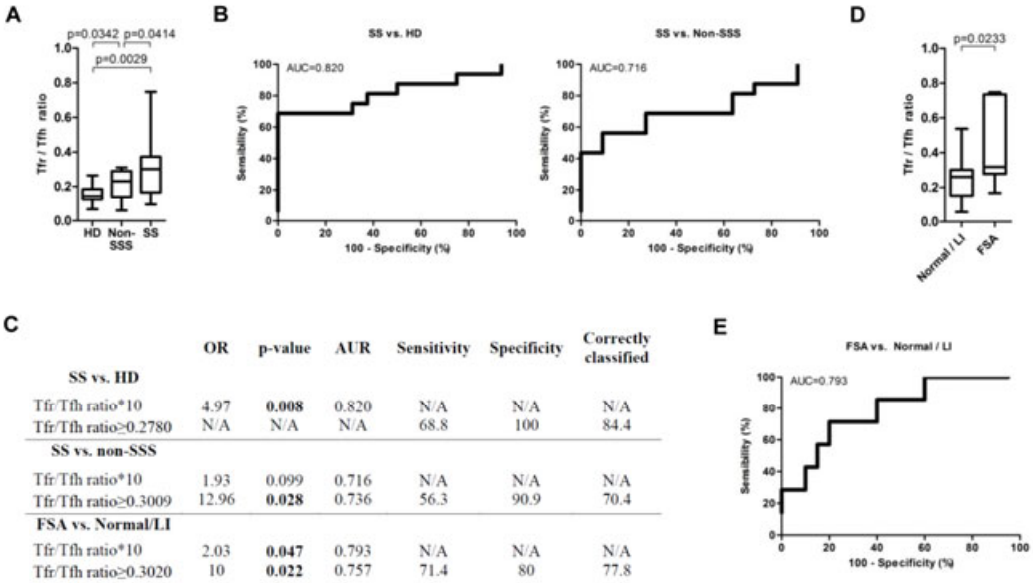

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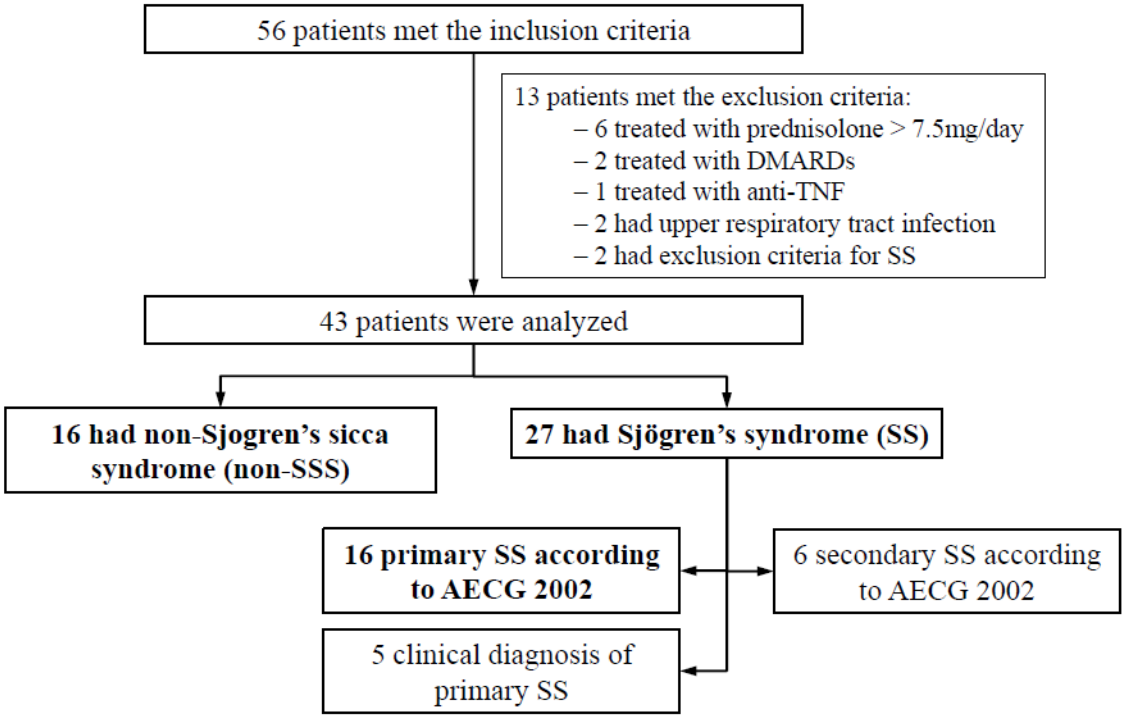
	OR	p-value	AUR	Sensitivity	Specificity	Correctly classified
SS vs. HD						
Tfr/Tfh ratio*10	4.97	**0.008**	0.820	N/A	N/A	N/A
Tfr/Tfh ratio≥0.2780	N/A	N/A	N/A	68.8	100	84.4
SS vs. non-SSS						
Tfr/Tfh ratio*10	1.93	0.099	0.716	N/A	N/A	N/A
Tfr/Tfh ratio≥0.3009	12.96	**0.028**	0.736	56.3	90.9	70.4
FSA vs. Normal/LI						
Tfr/Tfh ratio*10	2.03	**0.047**	0.793	N/A	N/A	N/A
Tfr/Tfh ratio≥0.3020	10	**0.022**	0.757	71.4	80	77.8



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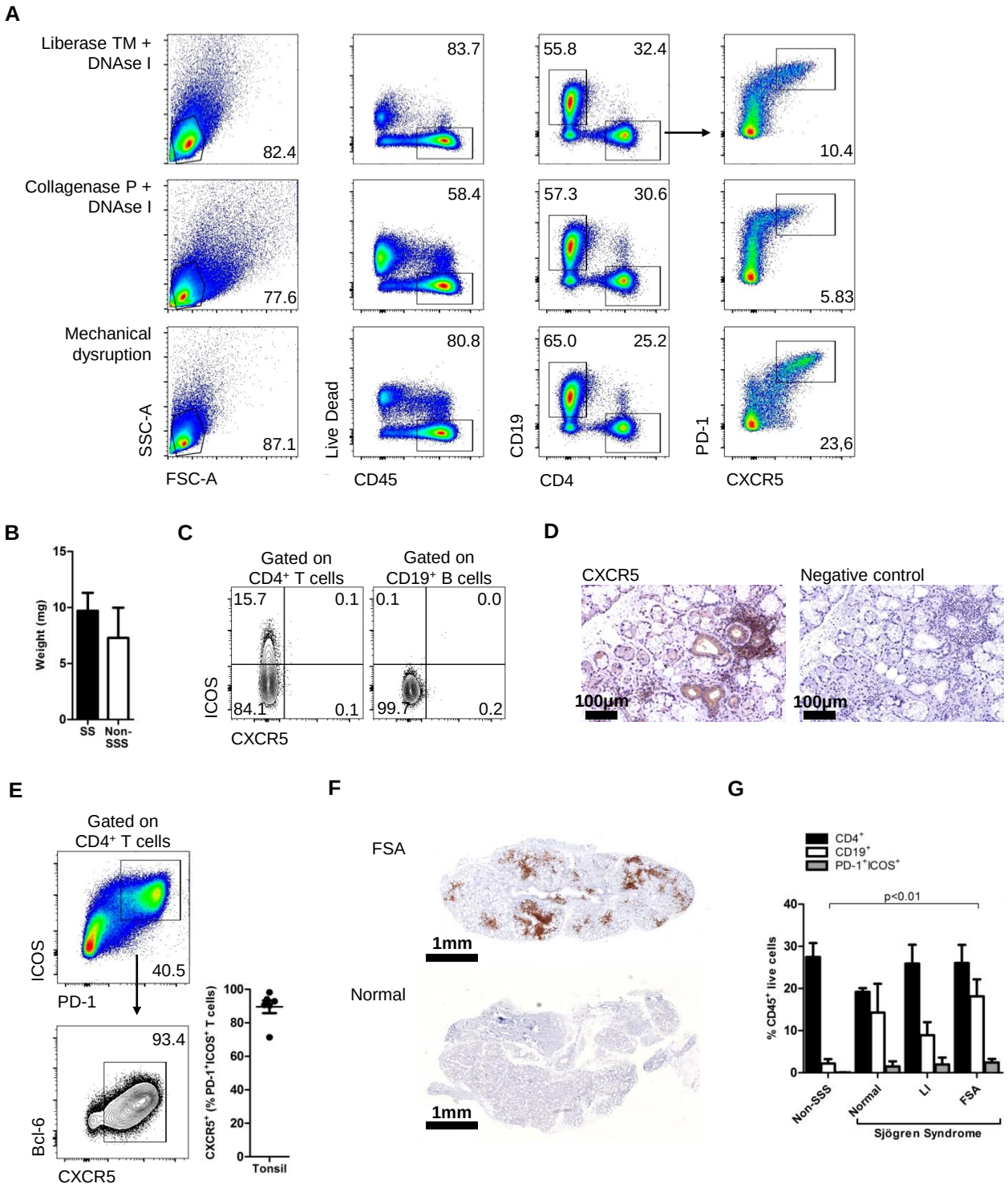

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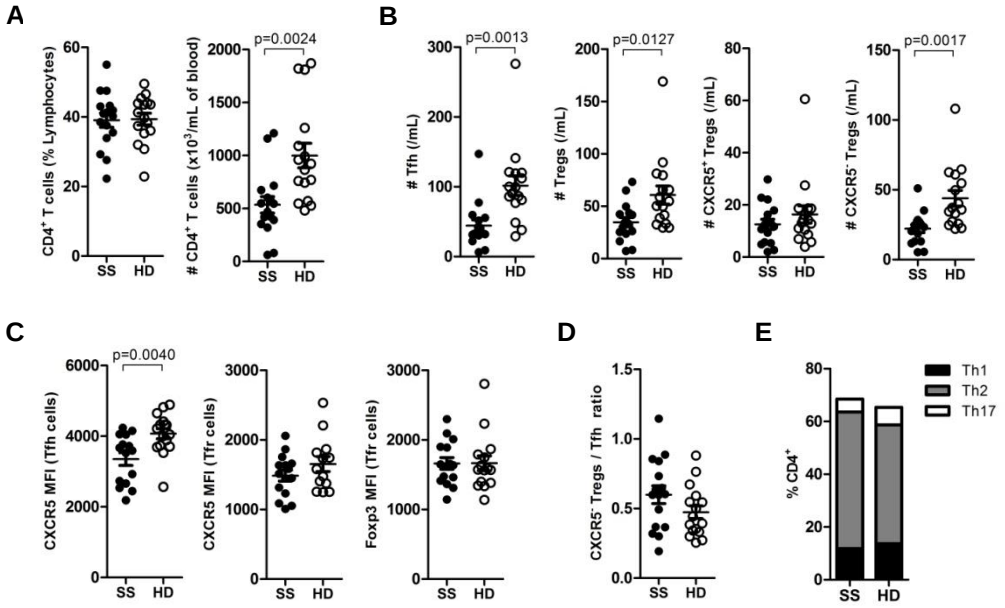

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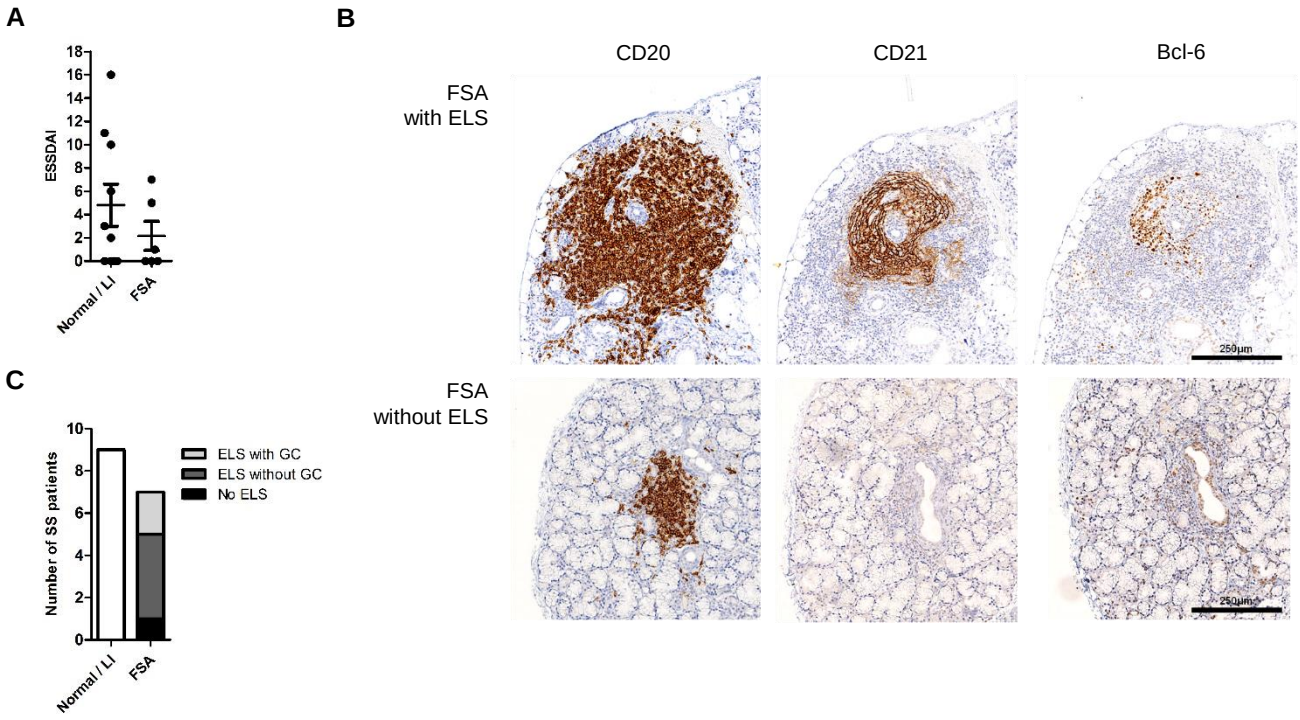

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Supplementary Table 1: Demographic and clinical characteristics of primary Sjögren's syndrome (SS) and non-Sjögren sicca syndrome (non-SSS) patients.

Patient	Gender, Age	Diagnosis	ESSDAI	ANA	Anti-Ro52	Anti-Ro60	Anti-La	RF	γ-fraction	ESR	CRP	SG Biopsy	Treatment
1	F, 50	pSS	10	1/640	59	13748	17069	16	2.4	85	2.29	LI	-
2	F, 27	pSS	16	1/1280	3392	13748	129	49	1.8	30	0.37	LI	-
3	F, 66	pSS	0	1/320	1024	13748	223	<14	1.5	89	1.18	FSA	-
4	F, 57	pSS	0	1/640	0	0	0	<14	1.1	12	0.04	FSA	-
5	F, 62	pSS	0	0	722	9761	741	<14	1.1	24	0.05	Normal	HCQ
6	F, 33	pSS	0	1/160	478	0	0	<14	1.8	47	0.2	Normal	-
7	F, 67	pSS	1	1/320	44	5666	0	<14	1.4	62	0.86	FSA	-
8	F, 17	pSS	3	1/640	8483	13748	515	30.8	1.9	27	0.34	Normal	-
9*	F, 51	pSS	0	1/160	+	+	+	<14	1.5	NA	NA	LI	PDN
10	F, 49	pSS	7	1/320	5898	13748	184	31.9	2.9	70	0.44	FSA	HCQ, PDN
11	F, 46	pSS	0	1/320	476	3095	0	60.2	1.2	30	0.22	FSA	-
12	F, 78	pSS	5	1/640	3587	13748	374	101.2	NA	30	0.34	FSA	HCQ, PDN
13	M, 40	pSS	2	1/640	1437	13748	34	<14	1.5	22	0.05	LI	-
14	F, 79	pSS	6	1/160	58985	13748	0	44.8	2.1	69	0.76	LI	-
15	F, 69	pSS	11	1/320	27785	0	0	31.6	2.8	47	0.39	LI	PDN
16	F, 45	pSS	0	1/640	12810	11138	29	<14	1.3	12	0.43	FSA	PDN
17	F, 29	Non-SSS	-	0	0	0	0	<14	NA	NA	NA	Normal	-
18	F, 26	Non-SSS	-	0	0	0	0	<14	0.8	9	0.72	Normal	-
19	F, 32	Non-SSS	-	0	0	0	0	<14	0.9	15	0.41	Normal	-
20	F, 72	Non-SSS	-	0	0	0	0	<14	1.5	59	0.13	Normal	-
21	F, 36	Non-SSS	-	0	0	0	0	<14	1.7	26	0.04	Normal	-
22	F, 52	Non-SSS	-	0	0	0	0	NA	1.0	5	0.05	LI	PDN
23	F, 39	Non-SSS	-	0	0	0	0	18.7	NA	18	0.04	LI	-
24	F, 45	Non-SSS	-	0	0	0	0	<14	1.2	25	NA	Normal	-
25	M, 59	Non-SSS	-	0	0	0	0	<14	0.7	3	0.04	Normal	-
26	F, 61	Non-SSS	-	0	0	0	0	<14	0.8	3	0.81	Normal	-
27	F, 48	Non-SSS	-	0	0	0	0	<14	NA	29	0.04	Normal	-
28	F, 30	Non-SSS	-	0	0	0	0	<14	1.1	18	0.06	Normal	-
29	F, 60	Non-SSS	-	0	0	0	0	<14	0.9	16	0.08	LI	-
30	F, 52	Non-SSS	-	1/160	0	0	0	<14	1.2	29	0.27	Normal	-
31	F, 61	Non-SSS	-	1/80	0	0	0	<14	NA	25	2.28	LI	-
32	F, 68	Non-SSS	-	0	0	0	0	<14	NA	52	2.25	LI	-

Legend: CRP, C-reactive protein (mg/dl); ESSDAI, EULAR Sjögren's syndrome disease activity index; ESR, erythrocyte sedimentation rate (mm); F, female; FSA, focal sialadenitis; HCQ, hydroxychloroquine; LI, unspecific lymphocytic infiltration; M, male; NA, not available; non-SSS, Non-Sjögren's sicca syndrome; PDN, prednisolone; pSS, primary Sjögren's syndrome; RF, rheumatoid factor (UI/mL); SG, salivary gland; γ-fraction, serum electrophoresis gamma-fraction (g/dL). *Antibody titers not available.

Antibodies	Clone	Company
Anti-Bcl-6	K112-91	BD Biosciences
Anti-CCR6	G034E3	BioLegend
Anti-CCR7	#150503	R&D Systems
Anti-CD19	HIB19	BioLegend
Anti-CD25	BC96	eBioscience
Anti-CD3	OKT3	eBioscience
Anti-CD38	HB-7	BioLegend
Anti-CD4	OKT4	BioLegend
Anti-CD45	HI30	BioLegend
Anti-CD45RO	UCHL1	BioLegend
Anti-CXCR3	G025H7	BioLegend
Anti-CXCR5	J252D4	BioLegend
Anti-Foxp3	PCH101	eBioscience
Anti-ICOS	C398.4A	BioLegend
Anti-PD-1	EH12.2H7	BioLegend

Supplementary Table 2: Antibodies used for flow cytometry.

	Pearson, r	p-value
Clinical and laboratory parameters		
ANA	0.128	ns
Anti-SSA/Ro52, UQ	0.076	ns
Anti-SSA/Ro60, UQ	0.097	ns
Anti-SSB/La, UQ	-0.164	ns
RF, UI/mL	0.449	ns
γ-fraction, g/dL	0.601	0.018
CRP, mg/dL	-0.155	ns
ESR, mm	0.098	ns
ESSDAI	0.427	ns
Minor salivary gland populations		
$CD45^+$, cells /mg	0.360	ns
$CD4^+$, cells /mg	0.380	ns
$PD\text{-}1^+ICOS^+CD4^+$, cells /mg	0.821	0.007
$CD19^+$, cells /mg	0.296	ns

Supplementary Table 3: Correlation between absolute number of Tfr cells per mm^2 of minor salivary gland tissue section (Fig. 2F) wit clinical and laboratory parameters, and minor salivary gland infiltration by $CD45^+$, $CD4^+$, $CD19^+$ and $PD\text{-}1^+ICOS^+$ lymphocytes. (n=16, Pearson coefficient, r).

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IMMUNOLOGY REVIEW ARTICLE

T follicular regulatory cells in mice and men

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Summary

It has long been known that CD4 T cells are necessary to provide help to B cells, triggering a germinal centre (GC) reaction where affinity maturation and isotype switching occur. However, the nature of the dedicated CD4 helper T cells, known as T follicular helper (Tfh), was only recently described. Here, we review the biology and function of the recently described T follicular regulatory (Tfr) cells, another CD4 T-cell population also found within GCs but with regulatory function and characteristics. Tfr cells have been identified in mice and humans as simultaneously presenting characteristics of T follicular cells (namely CXCR5 expression) and regulatory T cells (including Foxp3 expression). These Tfr cells have been implicated in the regulation of the magnitude of the GC reaction, as well as in protection from immune-mediated pathology.

Keywords: autoimmunity; germinal centres; regulation; T follicular helper cells; T follicular regulatory cells.

Introduction

Germinal centres (GCs) are secondary lymphoid structures within B-cell follicles where B cells go through affinity maturation (somatic hypermutation and positive selection) and class-switch recombination to generate high-affinity antibodies.[1] For B cells to undergo these processes, they require the help of T follicular helper (Tfh) cells, a specialized $CD4^+$ T-cell subset that provides survival, proliferation and selection signals by engaging in cognate interactions with B cells.[2] Moreover, Tfh cells also produce important cytokines for the GC reaction, namely interleukin-21 (IL-21) and IL-4.[2] Another $CD4^+$ T-cell population has been recently described as present in GCs, the T follicular regulatory (Tfr) cell population. The Tfr cells are a subset of forkhead box P3 positive ($Foxp3^+$) regulatory T (Treg) cells that acquire a follicular phenotype and migrate into GCs, where they act as regulators of the GC reaction on multiple levels.[3–5]

The importance of Treg cells for the control of antibody responses has been long known. Indeed, one of the consequences of the absence of Treg cells is the increased level of circulating antibodies, namely IgG and IgE, which originate in GCs.[6,7] Further studies showed that Treg cells are capable of controlling antibody responses by inhibiting activation-induced cytidine deaminase expression and class-switch recombination, and by directly killing B cells.[8–10] Also, CXC chemokine receptor type 5 positive ($CXCR5^+$) Treg cells could be found within GCs of immunized mice.[11] However, the confirmation of the existence of a specialized subset of Treg cells, which migrates into GCs and controls antibody responses, came with the identification of the Tfr cell population by three independent groups.[3–5] These studies, performed in mouse models, described a population with mixed characteristics of Treg and Tfh cells.[3–5] Tfr cells express not only the Treg master regulator Foxp3 but also other Treg-related molecules such as CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-induced tumour necrosis factor receptor-related protein, and granzyme B. In addition, Tfr cells express Tfh cell-associated molecules including CXCR5, inducible T-cell

Abbreviations: Bcl-6, B-cell lymphoma 6; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CXCR5, CXC chemokine receptor type 5; DC, dendritic cell; Foxp3, forkhead box P3; GC, germinal centre; ICOS, inducible T-cell co-stimulator; IL, interleukin; NFAT2, nuclear factor of activated T cells 2; NP, 4-hydroxy-3-nitrophenyl; PD-1, programmed cell death 1; PD-L1, PD-1 ligand 1; Tconv, conventional $CD4^+$ T; TCR, T-cell receptor; Tfh, T follicular helper; Tfr, T follicular regulatory; TGF-β, transforming growth factor β; TRAF3, tumour necrosis factor receptor-associated factor 3; Treg, regulatory T

co-stimulator (ICOS), programmed cell death 1 (PD-1), signalling lymphocytic activation molecule-associated protein, and, importantly, the master transcription factor of both Tfh and Tfr cells B-cell lymphoma 6 (Bcl-6). Hence, Tfr cells present, simultaneously, the characteristics of both Treg and Tfh cells by maintaining a suppressive function and gaining ability to access B-cell follicles and GCs.

Tfr cells have been described in mice and humans: one of the first reports describing Tfr cells demonstrated the presence of these cells within GCs of human tonsils.[3] However, as for Tfh cells, the difficulty in obtaining human samples of secondary lymphoid tissues has led to most of the work being performed in mice. Nevertheless, several recent studies have investigated a population of $CXCR5^+$ $Foxp3^+$ $CD4^+$ T cells from human blood, considered to be circulating Tfr cells, in samples from patients with autoimmunity or infectious diseases.

The initial studies describing Tfr cells described several key characteristics of this population, namely Tfr ability to regulate GC reactions. Further studies, in mice and human samples, have complemented our knowledge regarding Tfr function, origin, repertoire and specificity, as well as the regulatory mechanisms employed. Nevertheless, as most of the studies have been performed in mouse models, whether human Tfr cells have the same characteristics awaits confirmation. A brief summary of the known characteristics and markers used to identify Tfr cells in mice and humans can be found in Table 1.

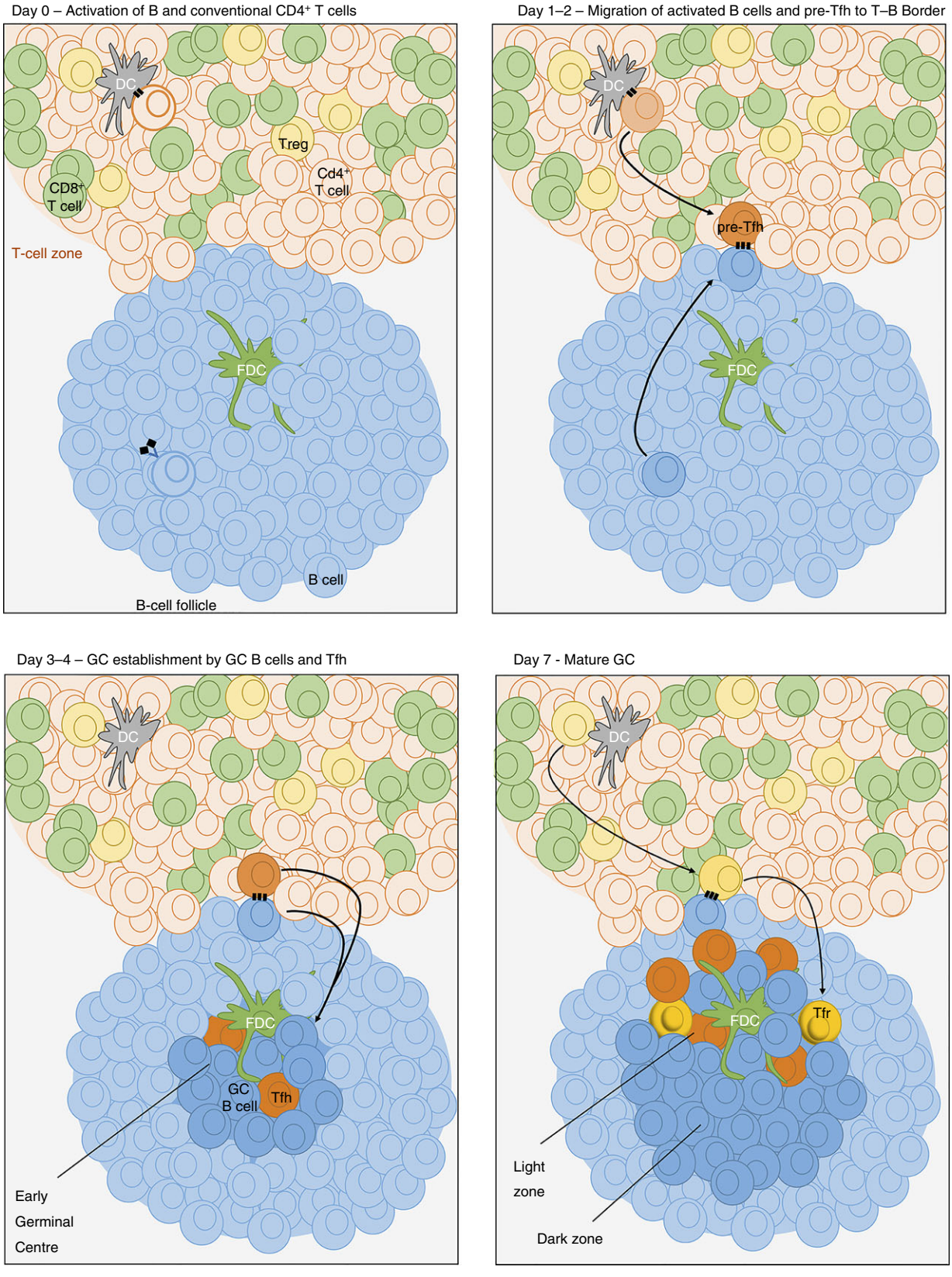
The biology of Tfr cells

The differentiation of Tfr cells is still not as characterized as the differentiation of Tfh cells. Nevertheless, Tfr cells seem to undergo a multi-step differentiation process similar to Tfh cells (Fig 1). For Tfh cells, such multistep priming is initiated, as for other $CD4^+$ T-cell subsets, following antigen recognition on dendritic cells. For the subsequent step, cognate interactions with B cells are required for full Tfh differentiation and expansion.[2,12,13] The same priming requirements were demonstrated for Tfr cells, which were substantially reduced in immunized mice where dendritic cells had been ablated.[14] The full differentiation of Tfr cells is also dependent on interactions with B cells. Indeed, Tfr cells are almost absent in draining lymph nodes of immunized mice that lack B cells.[14] However, these mice still contain a population of circulating Tfr cells upon immunization.[14] Hence, this population of blood Tfr cells originates upon the initial interaction with dendritic cells and before full commitment to the GC fate. The same study also showed that circulating Tfr cells can persist for long periods of time, representing a pool of cells that can later be recruited into the GC to suppress subsequent responses.[14]

Another similarity between the differentiation processes of Tfr and Tfh cells is the requirement of cognate signalling through the T-cell receptor (TCR) and co-stimulatory signals through CD28 and ICOS.[4,15–17] The CD28 requirement for Tfr cell commitment was first shown with bone marrow chimeras, containing a mixture of CD28-deficient and CD28-sufficient cells, where Tfr and Tfh cells originated exclusively from CD28-sufficient precursors.[4] These results were later corroborated by a study showing the absence of Tfr and Tfh cells in CD28-deficient and in ICOS-deficient mice.[17]

There are, however, some differences between the differentiation processes of Tfr and Tfh cells. A striking difference is that only the differentiation of Tfr cells seems to be affected by co-inhibitory signals. Although PD-1 is equally expressed by Tfr and Tfh cells, PD-1-deficient mice display an increased number of Tfr cells whereas the Tfh population remains unaffected.[17] The authors further established that the negative effect of PD-1 in Tfr cells is dependent on binding of PD-1 ligand 1 (PD-L1), but not PD-L2, since only PD-L1-deficient mice could replicate the Tfr phenotype observed in PD-1-deficient mice. Another inhibitory molecule that has a negative impact on Tfr cell differentiation and function is CTLA-4. Using mouse strains where CTLA-4 was conditionally deleted on $Foxp3^+$ Treg cells, two groups simultaneously demonstrated that, under those conditions, the suppressive capacity Tfr cells and their ability to control B-cell responses were diminished even though there was an increase of total Tfr cell numbers.[18,19] Similar results were reported following CTLA-4 targeting with a blocking antibody.[18] The cytokine IL-21 also has a negative impact on Tfr cell numbers even though it supports Tfh differentiation.[20,21] Interleukin-21 induces Bcl-6 expression, which, in turn, limits CD25. The reduction of CD25 expression then leads to lower responsiveness to IL-2 that, consequently, has a negative impact on the proliferation of Tfr cells.[20]

Figure 1. Dynamics of germinal centre (GC) development and T follicular regulatory (Tfr) cell recruitment. GC responses are initiated by antigen recognition by B cells within the follicle and $CD4^+$ T-cell priming by dendritic cells (DCs) in the T-cell zone. Within 1–2 days after activation, both activated B and pre-T follicular helper (Tfh) cells migrate into the T–B border where they engage in cognate interactions. Upon commitment to the GC programme, GC B and Tfh cells migrate into the centre of the follicle where an early GC is established between a network of follicular dendritic cells (FDCs). At day 7, the now mature GC has increased in size due to fast cell proliferation and it can be divided into two zones: the dark and light zones. The dark zone is mainly composed of rapidly dividing B cells, whereas in the light zone other cell types like Tfh and FDCs can be found. Tfr cells start to accumulate in the GC at this point, however, besides also requiring priming by DCs, the differentiation mechanism and required signals are still not well understood.


Day 0 – Activation of B and conventional CD4+ T cells
DC
Treg
Cd4+ T cell
CD8+ T cell
T-cell zone
FDC
B cell
B-cell follicle
Day 1–2 – Migration of activated B cells and pre-Tfh to T–B Border
DC
pre-Tfh
FDC
Day 3–4 – GC establishment by GC B cells and Tfh
DC
FDC
GC B cell
Tfh
Early Germinal Centre
Day 7 - Mature GC
DC
FDC
Tfr
Light zone
Dark zone

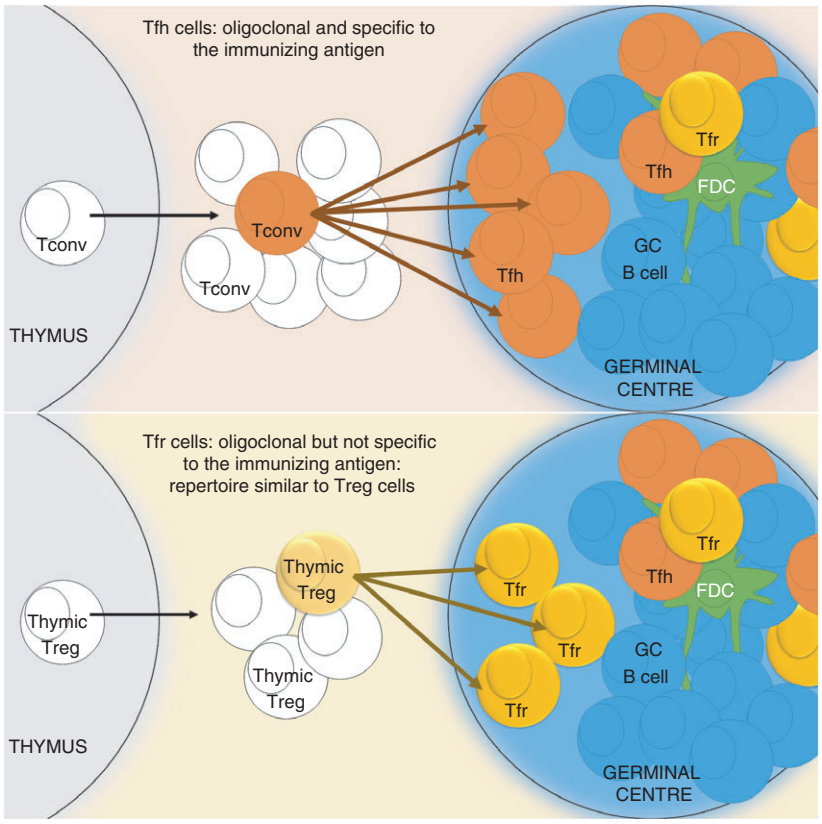


Figure 2. T follicular helper (Tfh) and T follicular regulatory (Tfr) cell ontogeny. While Tfh cells originate from conventional $CD4^+$ T (Tconv) cells, Tfr cells originate mainly from thymic regulatory T (Treg) cells. Moreover, although Tfr cells undergo some clonal expansion, their T-cell receptors (TCRs) are not specific for the non-self antigen driving the GC response. In fact, Tfr cells keep a TCR repertoire similar to that of Treg cells.

There are also similarities and differences in the transcription factors involved in Tfr and Tfh cell differentiation. As referred above, Bcl-6 is the master regulator for both subsets.[3,4,22] Signal transducer and activator 3 is also required by both Tfr and Tfh populations but, although Tfh numbers are reduced in mice where signal transducer and activator 3 was conditionally deleted on $CD4^+$ T cells, Tfr differentiation is almost abrogated in those mice.[23,24] A key difference on the transcription factors present in Tfr and Tfh cells is the balance between Bcl-6 and Blimp-1.[4] Whereas Tfh cells only express Bcl-6, Tfr cells express, simultaneously, both mutual antagonists and repressors Bcl-6 and Blimp-1.[4] It has been suggested that, while Bcl-6 is important to acquire a Tfh-like phenotype by Tfr cells, Blimp-1 may be necessary for the Treg-like suppressive function of Tfr cells.[4] Another difference is that CXCR5 expression in both subsets seems to have a distinct regulation. Tfh cells require achaete-scute homologue-2 for initial CXCR5 expression.[25] Tfr cells, however, do not express achaete-scute homologue-2 and appear to rely on nuclear factor of activated T cells 2 (NFAT2) to express CXCR5.[25,26] Indeed, NFAT2 was shown to bind to the CXCR5 promoter and induce its transcription, and Tfr cell numbers are reduced in NFAT2-deficient mice. In addition, the tumour necrosis factor receptor-associated factor 3 is only important for Tfr differentiation and function, and is involved in the regulation of ICOS in Treg cells.[27] Therefore, Treg-conditional tumour necrosis factor receptor-associated factor 3-deficient mice have impaired Tfr cell differentiation.[27]

The temporal accumulation of Tfr and Tfh cells in the GC is also different. Although the same type of cellular interactions seems to trigger both Tfr and Tfh cell differentiation, kinetic studies of the accumulation of both populations after immunization revealed that Tfr cells appear in the GC at later time-points, reaching their maximum frequency around 5 days later than Tfh cells do (Fig 1).[5,28] This unsynchronized accumulation of Tfr and Tfh cells may be required for the initial establishment of the GC, where few Tfh and GC B cells need to rapidly expand without the suppressive action of Tfr cells. On the contrary, once the GC has reached a significant size, Tfr cells would be important for the control of the GC reaction and its outcome.

The origin and specificity of Tfr cells are two other features that have been addressed in several studies. The first three studies describing Tfr cells reported that these cells derived from thymic Treg cells.[3–5] Linterman *et al.* observed that antigen-specific TCR-transgenic $CD4^+$ cells, which do not contain thymic Treg cells, did not differentiate into Tfr cells (even though they readily gave rise to Tfh cells).[4] Our group used T-cell-deficient mice as recipients of antigen-specific TCR-transgenic $CD4^+$ cells and thymic Treg cells to demonstrate that only the thymic

Table 1. Biology of T follicular regulatory (Tfr) cells in mice and humans

	Mice	Humans
Membrane markers	CD25, CXCR5, PD-1, ICOS	CD25, CXCR5, PD-1, ICOS[1]
Transcription factors	Foxp3, Bcl-6, Blimp-1	Foxp3, Bcl-6
Cell of origin	Thymic Treg cells	Not directly studied
Function	Control Tfh and GC B-cell numbers by inducing a suppressive state and inhibiting their proliferation; Impair IL-4 and IL-21 production by Tfh cells; Reduce the amount of antibodies produced (namely IgM and IgG) and the occurrence of class switch recombination; Control antibodies' quality by inducing production of high-affinity antibodies.	Impair Tfh cell proliferation; Impair IL-4 and IL-21 production by Tfh cells. Impair immunoglobulin (namely IgA) production by B cells.
TCR repertoire	Tfr cell repertoire is different from that of Tfh cells but similar to the repertoire of Treg cells.	Not studied
Immune compartments	Lymph node, spleen, blood[2]	Tonsil, lymph node, spleen, blood[2], ectopic lymphoid structures
Identification by flow cytometry	$CD4^+$ $Foxp3^+$ plus at least two of the following: $CXCR5^+$, $PD\text{-}1^+$, $ICOS^+$, $Bcl\text{-}6^+$	$CXCR5^+$ $Foxp3^+$ $CD25^+$ $CD4^+$ (for tissue Tfr cells use at least one of the following: PD-1, ICOS)
Identification by microscopy	$Foxp3^+$ cells within GC	$Foxp3^+$ cells within GC

[1]ICOS expression in humans does not discriminate Tfr cells from other effector Treg cells.
[2]The function and biological role of Tfr cells in peripheral blood is still elusive, especially in humans.

Treg cells could differentiate into Tfr cells.[5] Finally, Chung *et al.* also used T-cell-deficient mice as recipient, but co-transferred wild-type naive $CD4^+$ T cells and $Foxp3^+$ Treg cells with different congenic markers, and showed that Tfr cells originated from the Treg cell population.[3]

A recent report showed that, under specific conditions, Tfr cells can originate from naive conventional $CD4^+$ T cells and be specific for the immunizing antigen.[29] Indeed, in bone marrow chimeras, where only $CD4^+$ T cells devoid of thymic Treg cells can express CXCR5 and, consequently, in the absence of physiological competition with thymic Treg cells, some Tfr cells originated from those naive conventional T cells. However, we have recently reported that under physiological competition with thymic Treg cells, virtually all Tfr cells derived from the thymic Treg cell population (Fig 2).[30] Moreover, we found that GCs from immunized mice contained Tfh cells stained with an appropriate MHC class II tetramer, whereas Tfr cells from the same GCs did not bind the tetramer.[30] Indeed, we could not detect antigen-specific tetramer-positive Tfr cells in any of the conditions tested. Furthermore, sorted Tfr cells did not preferentially proliferate or survive *in vitro* with immunizing antigen signals when compared with a control antigen. Finally, we sequenced the TCR-α-chain (*TRA* gene) of Tfr, Tfh and Treg cell populations from immunized TCR-β-restricted mice. The repertoire analysis showed that, although Tfr cells are an oligoclonal population, they have a repertoire that resembles the repertoire of Treg cells, but that is different from that of Tfh cells.[30] Hence, our work showed that the TCR repertoire of Tfh and Tfr cells from the same GCs are considerably different:[30] Tfh cells bear TCRs specific for the immunizing antigen, but the TCR repertoire of Tfr cells lacks those antigen-specific clones while being similar to the predominantly self-reactive repertoire of thymic Treg cells (Fig 2).

Tfr cell regulatory mechanisms

Tfr cells constitute a regulatory T-cell subset specialized in the control of the GC response. The initial studies that described Tfr cells already showed that this population controls the GC size and the amount of antibodies produced.[3–5] Indeed, in the absence of Tfr cells, the size of the GC detected by immunofluorescence was increased,[5] as well as the total numbers of Tfh and GC B cells.[3–5] In the same line, the amount of antigen-specific IgM and IgG antibodies was higher in the serum of immunized mice lacking Tfr cells.[3,5]

Another feature that has been studied is the impact of Tfr cells in the affinity of the antibodies produced. Chung *et al.* reported that Tfr cells led to reduced antibody affinity.[3] This conclusion was obtained by observing lower levels of high affinity 4-hydroxy-3-nitrophenyl (NP)-specific antibodies in T-cell-deficient mice, immunized with NP conjugated to a protein, that had received wild-type $CD4^+$ naive T and Bcl-6-deficient $Foxp3^+$ Treg cells when compared with mice that had received wild-type $Foxp3^+$ Treg and naive $CD4^+$ T cells. Two other studies, however,

Table 2. Human studies that include analysis of Foxp3$^+$ T follicular regulatory (Tfr) cells

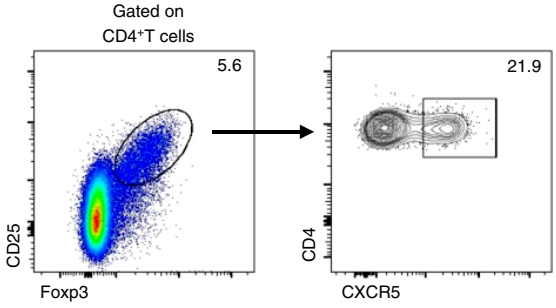
Ref.	Condition	Tissue	Tfr cells	Major findings
Faghih Z *et al.*[67]	Breast cancer	Lymph nodes	CXCR5$^+$ Foxp3$^+$ Bcl-6$^+$ CD4$^+$	No impact on Tfr cell frequency.
Wallin *et al.*[40]	Kidney transplant recipients undergoing therapy with rituximab	Lymph nodes	CD57$^+$ CXCR5$^+$ Foxp3$^+$ CD127$^-$ CD4$^+$	Rituximab had no impact on Tfr cells. Tfr cells reduce IgA production by B cells (*in vitro*).
Wang *et al.*[48]	Chronic hepatitis B (HBV) and C (HCV)	Blood	CXCR5$^+$ Foxp3$^+$ CD4$^+$	Tfr cells increased during HBV and HCV infection. Tfr cells positively correlate with HBsAg titres and viral load.
Chen *et al.*[49]	*Schistosoma japonica* infection	Blood	CXCR5$^+$ Foxp3$^+$ CD4$^+$	Tfr cell frequency increased during *S. japonica* infection.
Colineau *et al.*[63]	HIV	Spleen	Foxp3$^+$ CXCR5$^+$ CD45RA$^-$ CCR7$^-$ CD4$^+$	Tfr cell frequency increased during HIV infection.
De Bruyne *et al.*[53]	Food allergy	Blood	CXCR5$^+$ CD45RO$^+$ Foxp3$^+$ CD25$^+$ CD4$^+$	No impact on Tfr cell frequency.
Dhaeze *et al.*[50]	Multiple sclerosis (MS)	Blood	CXCR5$^+$ PD-1$^+$ CD25$^+$ CD127$^-$ CD4$^+$	Tfr cell frequency increased upon influenza vaccination, positively correlating with specific antibody titres. Tfr cell frequency was decreased in MS patients, showing less suppressive capacity.
Miles *et al.*[62]	HIV infection *In vitro*	Lymph nodes	CD25$^+$ CD127$^-$ CXCR5$^+$ CD3$^+$ CD8$^-$	Tfr cell expanded upon HIV infection (TGF-β dependent). Tfr cells reduced ICOS$^+$ IL-21$^+$ IL-4$^+$ Tfh cells.
Shan *et al.*[61]	Ankylosing spondylitis (AS)	Blood	CXCR5$^+$ Foxp3$^+$ CD4$^+$	Tfr cells were increased in AS, increasing further upon therapy. Tfr cells negatively correlated with serum IgA titres.
Di Fonte *et al.*[41]	PI3KR1 gain-of-function (c.1425 + 1G>T)	Tonsil	CXCR5$^+$ Foxp3$^+$ CD4$^+$	No impact on Tfr cell frequency.
Xu X *et al.*[69]	Kidney allograft rejection	Kidney	CXCR5$^+$ Foxp3$^+$	Tfr cells were rarely present in kidney tertiary lymphoid structures.
Wen *et al.*[52]	Myasthenia gravis (MG)	Blood	CXCR5$^+$ Foxp3$^+$ CD4$^+$	Tfr cells were decreased in MG, inversely correlating with disease severity. Treatment with steroids re-established the frequency of Tfr cells.
Vaeth *et al.*[26]	ORAI1 p.R91W	Blood	CD45RO$^+$ Helios$^+$ Foxp3$^+$ CD4$^+$	SOCE signalling defects impaired Tfr cell frequency.

associated impaired Tfr function with the production of antibodies with lower affinity.[19,31] Kawamoto *et al.* demonstrated that Tfr cells are required in GCs of Peyer's patches for the generation of high-affinity IgA antibodies, which in turn are essential for maintenance of normal and diverse microbiota.[31] The authors co-transferred naive CD4$^+$ T cells with Bcl-6-deficient or Bcl-6-sufficient CD25$^+$ Treg cells into T-cell-deficient mice, and verified that IgA

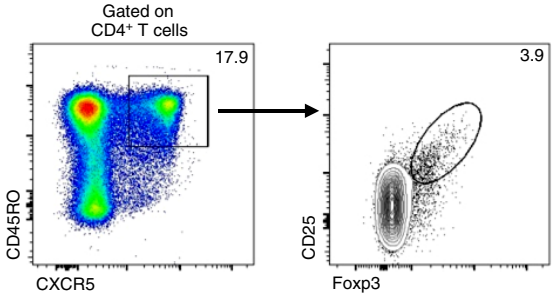
Box 1 $CXCR5^+Foxp3^+$ Tfr cells may affect the evaluation of other cell subsets

Many studies on T cell subsets in human blood have been performed without addressing Tfr cells as a separate population. This can influence the outcome of those studies.

1 Tfr cells are about 18·57 ± 6·55% of $CD25^+$ $Foxp3^+$ T cells (generally assumed to be Treg cells) in peripheral blood of healthy adults.



2 Tfr cells represent 4·01 ± 2·28% of $CD45RO^+$ $CXCR5^+$ T cells (generally assumed to be Tfh cells) in peripheral blood of healthy adults.



As a consequence, for adequate quantification of Tfh and Treg subsets, $CXCR5^+$ $Foxp3^+$ Tfr cells should be considered as a distinct cell population and studied separately.

produced in Peyer's patches from mice lacking Tfr cells had lower affinity and diversity. In the other study, Sage *et al.* showed that, when the action of Tfr cells is compromised by Treg-conditional deletion of CTLA-4, the amount of antibodies produced was higher, but the affinity generated (measured as the ratio between high- and low-affinity NP-specific antibodies on mice immunized with NP-ovalbumin) was lower.[19] This possible effect on antibody affinity could be explained by the facilitated help from Tfh cells to B cells in the absence of Tfr cells, and consequent generation of low-affinity plasma cells.[32]

The impact of Tfr cells on the GC reaction seems to be due to its direct action on Tfh and GC B cells. Tfh cells cultured in the presence of Tfr cells express lower levels of Ki-67 and produce less IL-4 and IL-21, indicating a suppressive impact of Tfr cells on Tfh proliferation and cytokine production.[14] In addition, in the absence of Tfr and Treg cells (by transiently depleting $Foxp3^+$ cells 6 days after immunization), there is a reduction of GC B cells specific for the dominant epitope of the immunizing antigen, so implicating Tfr cells in the regulation of non-antigen-specific B-cell clones in the GC.[4] Whether this impact was due to a direct effect of Tfr cells on GC B cells or a consequence of less regulation of Tfh clones by Tfr cells leading to greater 'uncontrolled' help to B cells was not addressed.

CTLA-4 is the only molecule expressed by Tfr cells so far described as a direct mediator of Tfr cell suppressive function.[18,19] As referred previously, in the absence of CTLA-4 on Treg cells, there is an increase in the Tfr cell numbers, but those Tfr cells lose most of their suppressive capacity.[18,19]

At least part of the suppressive mechanism of Tfr cells seems to involve the modulation of Tfh and GC B cells metabolism.[33] Indeed, in *in vitro* co-cultures, Tfr cells induce a suppressive state on Tfh and GC B cells, especially at metabolic level (i.e. glucose uptake, glycolysis and one-carbon metabolism), that persists in the absence of Tfr cells and is associated with epigenetic changes.[33] This suppressive effect translates in the inhibition of class-switch recombination and antibody production by B cells, and IL-21 and IL-4 production by Tfh cells. The suppressive state is reversible, as it can be abrogated in the presence of high levels of IL-21, which acts directly on both B cells (restoration of B-cell activation) and Tfr cells (inhibition of proliferation).[33] In fact, this observation is in line with IL-21 specifically rendering Tfr cells less responsive to IL-2, in both mice and humans, and, consequently, having a negative impact on the proliferation of Tfr cells.[20]

Despite the fact that most of the suppressive capacity of Tfr cells is lost in the absence of CTLA-4, it is expected that these cells employ multiple and complementary regulatory mechanisms, as has been described for Treg cells.[34–36] Several mechanisms have been proposed that involve: (i) the secretion of the regulatory cytokines IL-10 and transforming growth factor β (TGF-β); (ii) the induction of cell death by secretion of granzyme B; (iii) displacement of Tfh cells from GC B-cell surface; and (iv) interaction with Tfh and/or GC B cells through a still unknown receptor.[37]

Although IL-10 is regarded as a suppressive cytokine, evidence seems to exclude the production of IL-10 by Tfr cells as a regulatory mechanism of the GC reaction. Tfr cells produce IL-10, but high amounts of this cytokine were detected in the supernatants of Tfh and GC B-cell cultures when Tfr cells were not present.[14] Indeed, and contrary to what would be expected, Tfr cells seem to inhibit IL-10 production; however, IL-10 inhibition may still be in agreement with Tfr cell suppressive functions, as IL-10 is important for GC B-cell survival and proliferation.[14,38] The production of TGF-β may be an additional mechanism of Tfr suppression, as Tfh cells are suppressed by this cytokine.[39] Tfr cells also express granzyme B,

though in lower levels than Treg cells, and granzyme B-mediated cytolysis can be another regulatory mechanism employed by Tfr cells.[4]

Tfr cells in humans

Pioneering work from Lim *et al.* have established the existence of a CD69$^-$ human tonsil Treg cell subset with B-cell suppressive function.[8,9] Upon activation, this subset up-regulated CXCR5, acquiring the capacity to migrate towards CXCL13-enriched GC. In their *in vitro* assays, Lim *et al.* demonstrated that these cells suppressed class-switch recombination by B cells, as they inhibited immunoglobulin production and activation-induced cytidine deaminase expression. Those earlier observations gained a new importance following the identification of Tfr cells as a specialized cell subset.[3–5]

Since their discovery, Tfr cells have been regarded as putative important players in the pathogenesis of human diseases characterized by disrupted GC responses, like autoimmune and chronic infectious diseases (Table 2). The first evidence of Bcl-6$^+$ Tfr cells in humans was afforded by Chung *et al.* through the identification of tonsil CD4$^+$ T cells bearing the Foxp3$^+$ CXCR5$^+$ Bcl-6$^+$ phenotype.[3] As in mice, Tfr cells in human lymph nodes do not require B cells for their maintenance, as depletion of CD20$^+$ B cells by rituximab did not decrease the number of CD57$^+$ CXCR5$^+$ Foxp3$^+$ CD127$^-$ Tfr cells.[40] Similar to findings in mice, there is evidence suggesting some divergence in differentiation of human Tfr and Tfh cells: a primary immunodeficiency defined by PI3KR1 gain-of-function mutation did not affect the frequency of tonsil Tfr cells, but it significantly reduced the frequency of Tfh cells.[41]

Tfr cells, as well as Tfh cells, play their functional role in B-cell follicles and GC in secondary lymphoid tissues. The restricted access to human tissues forced the search for these cells in human blood. Several studies confirmed circulating CXCR5$^+$ T cells as the counterparts of tissue Tfh cells, arising from lymphoid tissue cells before reaching the GC, in spite of low Bcl-6 expression.[42–46] It is well known that some human blood Treg cells express CXCR5 among other CXC chemokine receptors.[47] Hence, many studies have been using circulating CXCR5$^+$ Foxp3$^+$ T cells to define Tfr cells in humans (Box 1).[48–53] Surprisingly, no study specifically addressed whether blood CXCR5$^+$ Foxp3$^+$ T cells are truly circulating Tfr cell counterparts. Although circulating memory Tfr cells were observed after immunization protocols in mice,[14,17] CXCR5 and Foxp3 transient up-regulation upon human T-cell activation challenged the assumption that human blood CXCR5$^+$ Foxp3$^+$ T cells are Tfr cells.[54–58] Dhaeze *et al.* found that blood CXCR5$^+$ PD-1$^+$ CD25$^+$ CD127$^+$ T cells increased on day 7 following influenza vaccination (showing a positive correlation with plasma antibody titres for influenza), and suppressed T cells *in vitro* upon αCD3/CD28 stimulation.[50] Although this report has correlated blood CXCR5$^+$ PD-1$^+$ CD25$^+$ CD127$^+$ T cells with influenza vaccination responses, it did not provide any evidence of an intrinsic humoral regulatory capacity. Our group has found that human circulating CXCR5$^+$ Treg cells are Tfr cell precursors that emerge after birth from lymphoid tissues as immature cells not yet endowed with full humoral regulatory function.[59] Our observation is in line with murine studies showing that circulating Tfr cells have less suppressive capacity than their tissue counterparts.[14,33]

Tfr cells in different human diseases

Autoimmune diseases

A disturbed B–T-cell interaction can be responsible for the generation of self-reactive antibodies in autoimmune diseases.[60] Although Tfh cells are indispensable for GC formation and generation of high-affinity antibodies, Tfh and Tfr cells are still poorly understood in human autoimmunity, mostly because of difficult access to the lymphoid tissues where those populations operate. Tfr cells are particularly attractive to study in the setting of autoimmunity as their modulation may have therapeutic potential.

Few studies have addressed Tfr cells in human autoimmunity (Table 2). Blood CXCR5$^+$ PD-1$^+$ CD25$^+$ CD127$^-$ Tfr cells were reduced in patients with multiple sclerosis.[50] In addition, blood Tfr cells from patients with multiple sclerosis were less suppressive than equivalent cells sorted from healthy controls, using CD25$^-$ CD127$^+$ T cells as responders *in vitro*.[50] This work suggests that blood Tfr cells might be functionally defective in autoimmunity. However, the functional assays performed in this study did not evaluate the putative specialization of Tfr cells in the suppression of humoral responses. An additional study also found a decreased frequency of blood CXCR5$^+$ Foxp3$^+$ Tfr cells in untreated patients with myasthenia gravis, recovering to normal levels after corticosteroid-based treatment.[52] However, not all studies of CXCR5$^+$ Foxp3$^+$ T cells in autoimmunity are concordant. A higher frequency of blood CXCR5$^+$ Foxp3$^+$ Tfr cells was found in patients with ankylosing spondylitis, increasing even further after treatment with Etanercept.[61] The blood frequency of Tfr cells did not correlate with disease severity of multiple sclerosis or ankylosing spondylitis, questioning the direct role of blood CXCR5$^+$ Foxp3$^+$ T cells in autoimmune pathogenesis. Besides organ-specific autoimmune diseases, it would be important to assess Tfr cells in systemic autoimmune diseases, where potentially more severe tolerance disruption mechanisms are operating. We found that blood Tfr cell frequency is increased in patients with Sjögren syndrome.[59] Therefore, our data suggest that blood Tfr cells,

as well as the Tfr : Tfh cell ratio, may constitute markers of disturbed GC responses in human autoimmunity.

HIV and other infectious diseases

During acute infection, a vigorous immune response leads to high-affinity antibody production within GCs. Although Tfr cell biology in humans remains largely uncharacterized, it is tempting to target Tfh and Tfr cells in conditions where antibody production is not sufficient to contain microorganism replication, like HIV infection.

HIV-1-infected individuals (not receiving highly active antiretroviral therapy) have an increased frequency of GC Foxp3$^+$ CD4$^+$ T cells,[62] spleen Foxp3$^+$ CXCR5$^+$ CD45RA$^-$ CCR7$^-$ CD4$^+$ Tfr cells,[63] and blood CXCR5$^+$ CD25$^+$ Foxp3$^+$ CD4$^+$ Tfr cells.[64] Tfh cells (defined as CXCR5$^+$ CD45RA$^-$ CCR7$^-$ CD4$^+$ T cells for spleen and as PD-1$^+$ CXCR3$^-$ CXCR5$^+$ CD4$^+$ T cells for peripheral blood) were also increased, suggesting that Tfr and Tfh cell expansion could occur simultaneously in cases of antigen persistence. Consistently, *in vitro* studies using tonsil cells spinoculated with X4 and R5 HIV have shown Tfr cell expansion (with increased CTLA-4, lymphocyte-activation gene 3 (LAG-3) and IL-10 expression) upon HIV infection in a TGF-dependent manner.[62]

In blood, the presence of broad neutralizing antibodies did not impact the frequency of Tfr cells, although patients with high titres of neutralizing antibodies displayed a higher expression of PD-1 in Tfr cells.[64] Although increased PD-1 signalling has been shown to inhibit Tfr cell function in mice,[17] it is still speculative to correlate the presence of broad neutralizing antibodies with putative Tfr cell exhaustion.

Blood CXCR5$^+$ Foxp3$^+$ Tfr cells were also found increased in hepatitis B virus and hepatitis C virus chronically infected patients, showing a significant correlation with blood viral load in both infections. An increased frequency of blood CXCR5$^+$ Foxp3$^+$ CD45RA$^-$ Tfr cells was also found in helminthic infection by *Schistosoma japonica*.[49]

Primary immunodeficiencies

Many human monogenic primary immunodeficiencies are associated with defective B-cell responses. In *STAT3*, *IL-10R*, *CD40L*, *NEMO*, *BTK* and *ICOS* mutations there is a decreased frequency of Tfh cells.[65] Although frequency of blood CXCR5$^+$ Foxp3$^+$ Tfr cells have not been studied in these pathological conditions, patients with $< 2\%$ of IgD$^-$ CD27$^+$ B cells in the setting of common variable immunodeficiency have a reduction of blood CXCR5$^+$ CD25^{hi} CD127^{low} Tfr cell frequency, in line with a reduction of total Treg cell frequency in peripheral blood.[66] This study suggests a relationship between this B-cell subset and blood Tfr cells, but the clinical heterogeneity and largely unknown molecular mechanisms driving common variable immunodeficiency preclude a definite conclusion about blood Tfr cell ontogeny. Recently, the SOCE (store-operated calcium entry) pathway in T cells has been implicated in Tfr cell differentiation in humans, as patients with severe combined immunodeficiency-like disease due to inherited loss-of-function mutations in *ORAI1* and *STIM1* genes that abolish SOCE have a significant reduction in blood CD45RO$^+$ Helios$^+$ Foxp3$^+$ Tfr-like cells.[67] In another recent study, IL-21R-deficienct patients have been shown to have a significant increase in frequency of blood Foxp3$^+$ CXCR5$^+$ PD-1$^+$ Tfr cells. In contrast, a marked decrease in circulating CXCR5$^+$ PD-1$^+$ Tfh cells was observed in IL-21R-deficiency patients.[20] Taken together, these recent studies suggest that human Tfh and Tfr cells have different, sometimes reciprocal, requirements for their differentiation. Therefore, the impact of the IL-21–IL-2 axis in Tfh and Tfr balance deserves further investigation, as its modulation may influence the outcome of GC responses.

Conclusions

The GC reaction is a key event in humoral responses. The B-cell–Tfh cell interactions are important for the production of high-affinity protective antibodies, following B-cell receptor hypermutation and selection. However, immune dysregulation may lead to autoantibody production and autoimmunity. The understanding of the interplay of Tfr, Tfh and GC B cells may lead to more effective vaccines and to novel therapeutic strategies for immune-mediated diseases.

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Disclosures

The authors declare no conflicts of interest.

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